Universidade de Aveiro Secção Autónoma de Ciências da Saúde

2015

PATRÍCIA DANIELA OLIVEIRA FERREIRA

REGULATION OF IRON METABOLISM IN DIFFERENT BACTERIAL INFECTIONS

REGULAÇÃO DO METABOLISMO DO FERRO EM DIFERENTES INFEÇÕES BACTERIANAS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Ana Carolina dos Santos Moreira, Investigadora de Pós-Doutoramento no Instituto de Biologia Molecular e Celular da Universidade do Porto e co-orientação da Doutora Maria de Fátima Matos Almeida Henriques de Macedo, Professora auxiliar convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro e investigadora no Instituto de Biologia Molecular e Celular da Universidade do Porto.

Dedico este trabalho aos meus avós

o júri

presidente

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agradecimentos

Todo o meu percurso académico, bem como o desenvolvimento desta tese não teriam sido possíveis sem o apoio de muitas pessoas às quais agradeço:

Ao IBMC e à UA por me deixarem fazer parte da sua grande família.

Ao Professor Drº Pedro Rodrigues por me ter dado a oportunidade de realizar a tese de mestrado no seu grupo de investigação.

À minha orientadora, Drª Ana Carolina Moreira, por ter sido incansável, por toda a paciência e disponibilidade, por ter acompanhado o meu trabalho desde do início, por me acalmar em momentos menos bons, por me dar motivação e me transmitir o seu positivismo e conhecimento.

À minha co-orientadora, Drª Fátima Macedo, por estar sempre pronta a ajudar.

À professora Salomé Gomes por estar sempre disponível para esclarecer qualquer dúvida e pelo apoio nas experiências práticas.

Ao Drº João Neves, pelo apoio nas experiências práticas e protocolos, pela disponibilidade em ajudar nos dias mais longos e pela boa disposição.

Ao biotério do IBMC, por estarem sempre disponíveis.

Ao Drº Didier Cabanes e à Drª Luísa Peixe por nos terem cedido as estirpes de *L. monocytogenes* e de *S*. Typhimurium, respetivamente.

À Drª Graça Henriques do Corelab por se dispor a receber e processar as amostras de sangue das experiências.

Aos meus colegas do laboratório, Tânia Silva, Ana, Tânia Magalhães, Inês, Tânia Moniz e Miguel, por me terem recebido de braços abertos, pelo bom ambiente no laboratório e por terem partilhado comigo o vosso conhecimento. Às meninas agradeço a companhia nas horas de almoço.

A todos os meus colegas de CBM e de MBM, em especial à Vanessa, à Andreia Rocha, à Cátia e à Raquel, por me terem aturado e por terem feito com que este percurso fosse mais fácil de percorrer.

Aos professores da UA pelos conhecimentos que me transmitiram.

Às minhas amigas, Dianne e Ana Sofia e ao meu namorado, pelo apoio, paciência e por todos os ”não posso tenho que estudar” que ouviram.

À minha prima Sandra por estar sempre disponível para me ajudar com os apontamentos, aulas e trabalhos.

Aos meus pais, os meus grandes pilares, a quem devo tudo o que sou e tudo o que tenho. Sem o apoio deles eu não teria conseguido.

O meu sincero obrigado.

palavras-chave Infeção bacteriana, *Listeria monocytogenes*, *Salmonella* Typhimurium, sistema imunitário inato, metabolismo do ferro, hepcidina, transferrina, ferritina, Interleucina-6, ferroportina

resumo

O ferro é encontrado em quase todos os seres vivos, desempenhando um papel central nas interacções entre o hospedeiro e o patógeno e sendo essencial para ambos. Para o hospedeiro, o ferro é um elemento crucial, uma vez que desempenha um papel chave em processos biológicos como o transporte de oxigénio, a biossíntese de DNA, produção de energia e regulação da expressão génica. No entanto, elevadas concentrações de ferro também podem ser tóxicas para as células devido à capacidade de gerarem radicais hidroxilo. Assim, os vertebrados possuem proteínas para transportar e armazenar o ferro, a transferrina e a ferritina respetivamente. A hepcidina é uma proteína chave do metabolismo do ferro, uma vez que se liga à ferroportina, o exportador do ferro, regulando a libertação de ferro para o soro. Por outro lado, o ferro é também fundamental para os patógenos, que o requerem para o seu crescimento e proliferação, para a expressão de factores de virulência e para vários processos metabólicos. Assim, durante a infecção, o hospedeiro e o patógeno competem por este metal. Os patógenos desenvolveram múltiplas estratégias para adquirir o ferro a partir do hospedeiro durante a infeção. Deste modo, tornar o ferro indisponível para os microrganismos é um mecanismo central na defesa do hospedeiro.

Neste trabalho, investigámos a regulação do metabolismo do ferro no hospedeiro durante a infecção com *Listeria monocytogenes*, uma bactéria gram-positiva e com *Salmonella* Typhimurium, uma bactéria gram-negativa, de modo a verificar se existem alterações no metabolismo do ferro do hospedeiro dependendo do tipo de infeção e se a hepcidina tem um papel preponderante nestas alterações.

Murganhos machos C57BL6 foram infectados com 10^4 CFU de *L. monocytogenes*, *S.* Typhimurium, ou um volume equivalente de veículo e sacrificados a diferentes tempos experimentais. A quantificação da carga bacteriana, determinação do ferro não hémico no fígado, avaliação da distribuição de ferro no tecido, análise histopatológica e a expressão de genes relacionados com o metabolismo do ferro foram analisados.

Os nossos resultados mostram que tanto na infeção com *L. monocytogenes* como na infeção com *S*. Typhimurium, o sistema imunitário do hospedeiro não é capaz de irradiar a infeção e, assim, a carga bacteriana aumenta durante a experiência. Em relação aos parâmetros hematológicos e serológicos, é observada a redução da quantidade de eritrócitos e do hematócrito, bem como dos níveis de ferro no soro. Os níveis de interleucina-6 e de hepcidina aumentam em diferentes tempos experimentais em cada infeção. Adicionalmente, a concentração de ferro não hémico aumenta no fígado durante a infeção com ambos os patógenos. Foram também detetadas alterações histopatológicas aquando da infeção com *L monocytogenes* e *S.* Typhimurium.

Os nossos dados sugerem que ambas as infeções induzem alterações no metabolismo do ferro do hospedeiro. Contudo, a infeção com *S*. Typhimurium parece ter efeitos mais precoces e mais severos no hospedeiro do que a infeção com *L. monocytogenes*.

Keywords bacterial infection, *Listeria monocytogenes*, *Salmonella* Typhimurium, innate immune system, iron metabolism, hepcidin, transferrin, ferritin, Interleukin-6, ferroportin

abstract

Iron is found in almost all living organisms, playing a central role in host-pathogen interactions and being crucial for both host and pathogens. In the host, iron is a crucial element, since it plays a key role in biological processes such as oxygen transport, biosynthesis of DNA, energy production and regulation of gene expression. However, high concentrations of iron can also be toxic to cells due to the ability to generate hydroxyl radicals. Thus, vertebrates developed proteins to transport and store iron: transferrin and ferritin, respectivetly. Hepcidin is a key protein of iron metabolism, since it binds to ferroportin, the iron exporter, regulating the release of iron to the serum. On the other hand, iron is also fundamental for pathogens that required it to its growth and proliferation, to the expression of virulence factors and to metabolic processes. Thereby, during infection, the host and the pathogen compete by this metal. Pathogens developed multiple strategies to acquire iron from the host during infection. Thus, making iron unavailable for microorganisms is a central mechanism in host defense.

In this work, we investigated the regulation of iron metabolism in host during infection with *Listeria monocytogenes,* a gram-positive bacterium and *Salmonella* Typhimurium, a gram-negative bacterium in order to verify whether there are alterations in host iron metabolism depending of infection type and if hepcidin have a central role in these alterations.

C57BL6 male mice were infected with 10^4 CFU of *L. monocytogenes*, *S.* Typhimurium, or an equivalent volume of vehicle and sacrificed at different time points. Bacterial load quantification, non-heme iron determination in liver, evaluation of iron distribution in tissue, histopathologic analyses and the expression of genes related with iron metabolism were analyzed.

Our results show that in both infections with *L. monocytogenes* and *S*. Typhimurium the host immune system are not able to irradiate the infection and, thus, the bacterial load increases during the experiment. Regarding the hematological and serological parameters, a reduction of red blood cells and hematocrit is observed, as well as, of serum iron levels. The levels of interleukin-6 and hepcidin increase at different time points in each infection. Additionally, non-heme iron concentration increases in liver during infection with both pathogens. Histopathological alterations were also detected during infection with *L monocytogenes* and *S.* Typhimurium.

Our data suggests that both infections induce alterations in host iron metabolism. However, the infection with *S*. Typhimurium appears to have earlier and more severe effects in the host than infection with *L. monocytogenes*.

CONTENTS

FIGURES AND TABLES

ABBREVIATIONS

Ab	Antibody
ABC	ATP-binding cassette
ActA	Actin assembly inducing protein
Ag	Antigen
ANG4	Angiogenin 4
AP-1	Activator protein 1
APCs	Antigen-presenting cells
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
CARD	Caspase activating and recruitment domain
CTL	Cytotoxic T lymphocyte
CSF-1	Colony-stimulating factor-1
DCs	Dendritic cells
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DCYTB	Duodenal cytochrome-b-like reductase
Ent	Enterobactin
EPO	Erythropoietin
ERFE	Erythroferrone
FasL	Fas ligand
Fc	Fragment crystallisable
Fe	Iron
FPN	Ferroportin
Fur	Ferric uptake regulator
GDBS	Glucosylated 2,3-dihydroxybenzoylserine
GDF15	Growth differentiation factor 15
GI	Gastrointestinal
Hamp 1	Hepcidin antimicrobial peptide 1
HCP-1	Haem carrier protein 1
HFE	Human hemochromatosis
HIF	Hypoxia-inducible factor
HJV	Hemojuvelin
HO-1	Haem oxygenase 1

HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HREs	Hypoxia-responsive elements
Hrt	Heme-regulated transporter
ICAM-1	Intercellular adhesion molecule-1
iE-DAP	Y-D-glutamyl-meso-diaminopilemic acid
IFN-γ	Interferon gamma
Ig	Immunoglobulins
IL	Interleukin
In	Internalin
iNOS	inducible nitric oxide synthase
IREs	Iron response elements
IRPs	Iron regulatory proteins
JAK	Janus kinase
Lcn2	Lipocalin 2
LcnR	Lipocalin 2 receptor
LLO	Listeriolysin
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LTA	Lipoteichoic acid
MAC	Membrane attack complex
MBL	Mannan binding lectin
MCH I	Major histocompatibility complex class I
MCH II	Major histocompatibility complex class II
MCP-1	Monocyte Chemoattractant Protein-1
MDP	Muramyl dipeptide
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa B
NK	Natural killer
NLRs	Nucleotide-binding oligomerisation domain like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
Nramp	Natural resistance-associated macrophage protein
NTBI	Non transferrin bound iron

PAMPs	Pathogen-associated molecular patterns
PG	Peptidoglycan
PRRs	Pathogen recognition receptors
RBCs	Red blood cells
RER	Rough endoplasmic reticulum
RIP2	Receptor-interacting protein 2
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RT-PCR	Real Time polymerase chain reaction
TBI	Transferrin bound iron
TCR	T cell receptor
TF	Transferrin
TFR	Transferrin receptor
TGF-β	Transforming growth factor beta
Th1	T helper 1 cells
Th2	T helper 2 cells
TIR	Toll-Interleukin receptor
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
TWSG1	Twisted gastrulation BMP signalling modulator 1
SCV	*Salmonella* containing vacuoles
SPI-1	*Salmonella* pathogenicity Island 1
sPLA2	Secretory phospholipase A2
STAT	Signal transducer and activator of transcription

I. INTRODUCTION

1. HOST DEFENSE MECHANISMS AGAINST BACTERIAL INFECTION

Humans are in continuous associations with microorganisms. Since different parts of the body communicate with the exterior, human body becomes a potential site of microbial entry. However it is relatively rare that these microorganisms cause damage to their host. In part, this is due to the effectiveness of the host defense mechanisms against these microorganisms, which restrict bacterial invasion and protect the host (Male et al. 2006, Kindt et al. 2007).

Host defense mechanisms are mediated by the immune response, which is composed of two major systems: the innate and the adaptive immune systems. The innate immune system is the primary defense mechanism against invading organisms, while the adaptive immune system acts as a second line of defense, being dependent of the antigens recognition by antibodies and being involved in generation of immunological memory (Akira et al. 2006, Kindt et al. 2007).

2. INNATE IMMUNE SYSTEM

The innate immune system includes defenses that are constitutively present, are mobilized immediately upon infection and reacts similarly to a variety of organisms, therefore it is not specific. The innate immune system is mainly comprised by three main components: (Basset et al. 2003, Kindt et al. 2007)

1) Mechanical component consisting of the physical barriers of the skin and mucosa, along with physiological functions such as cilial action, motility, desquamation and mucus secretion. An example is the dermis of the skin, which is composed by connective tissue, blood vessels, hair follicles, sebaceous glands, and sweat glands. The sebaceous glands are associated with the hair follicles and produce an oily secretion called sebum which maintains the acidic pH inhibiting the growth of most microorganisms.

2) Chemical component that can be divided into three subcomponents:
    a) Pattern recognition molecules;
    b) Proteins or peptides that kill microbes, such as complement.
    c) Cytokines and chemokines that orchestrate the immune response.

3) Cell component, which includes epithelial cells, mast cells, dendritic cells (DCs), phagocytic cells, such as macrophages and neutrophils, and natural killer (NK) cells.

Interaction of the host with the pathogen occurs at three levels: extra-epithelial, epithelial and sub-epithelial level. The outer, the extra-epithelial defense barriers consist of a variety of antibacterial substances that kill or inhibit the growth of bacteria, including enzymes, such as lysozyme, which hydrolyses the cell wall of bacteria; mucus and motility, which traps and removes the bacteria and IgM of limited antigen specificity, which traps the invading pathogen, and secretes anti-microbial peptides that induce the death of some pathogens (Basset et al. 2003, Kindt et al. 2007). Additionally, the commensal microbial flora is also included in the extra-epithelial innate defenses. This condition provides resistance to colonization either by occupation of potential binding sites or by secretation of inhibitory compounds (Basset et al. 2003).

Defense at the epithelial barrier includes the mechanical aspect of preventing the penetration by microorganisms. However, the binding of the pathogen to the epithelium triggers a series of alarm signals resulting in the secretion of chemokines that ultimately will be important for the recruitment of other components of the innate defense network, leading to the development of an acute inflammatory reaction in which there is an increase in vascular permeability that, in turn, leads to the extravasation of acute phase proteins and complement into the infected tissue (Basset et al. 2003).

2.1 PATHOGEN RECOGNITION

During infection, the innate immune system of the host recognizes pathogens by pattern recognition receptors (PRRs). Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain (NOD) like receptors (NLRs) represent two classes of PRRs in mammals that are able to recognize different microbial components, known as pathogen-associated molecular patterns (PAMPs) (Philpott and Girardin 2004, Akira et al. 2006, Kindt et al. 2007).

Pathogen-associated molecular patterns are crucial for survival of infectious agents which may express many different PAMPs at the same time. Different PRRs react with specific PAMPs, showing distinct expression patterns, activating specific signaling pathways, and leading to different antipathogen responses (Athman and Philpott 2004, Philpott and Girardin 2004, Akira et al. 2006, Kindt et al. 2007).

Toll-like receptors are transmembrane glycoproteins characterized by extracellular domains containing various leucine-rich-repeats (LRR) motifs with the ligand binding site and a cytoplasmic domain, termed the toll/IL-1R homology (TIR) domain (Akira et al. 2006).

Toll-like receptors recognize microbial structures in the earliest phase of the host defense response and are expressed in several immune cells, including antigen-presenting cells

(APCs), such as macrophages and dendritic cells (DCs), in B cells and specific types of T cells. There are 13 different TLRs in mammals that may be expressed either on the cell outer membrane, such as TLRs 1, 2, 4, 5, 6 and 10 or in intracellular compartments such as endosomes or lysosomes, namely TLRs 3, 7, 8, 9, 11 and 13 (Akira et al. 2006, Broz et al. 2012). The table 1 shows different classes of TLRs and PAMPs recognized by these receptors in different kinds of bacteria (Akira et al. 2006). It should be taken into account that in this table the TLRs only present in viruses, namely TLR3, TLR7 and TLR8, are not shown.

Table 1: TLRs classes and PAMPs recognized by these receptors in different bacteria species - Adapted from (Akira et al. 2006)

Microbial Components	Species	TLR Usage
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B *Streptococcus*	TLR6/TLR2
PG	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND	Uropathogenic bacteria	TLR11

Legend: LPS – Lipopolysaccharide; LTA - Lipoteichoic acid; PG – Peptidoglycan; ND – Not defined; TLR – Toll like receptor

After the ligand binding to the extracellular portion of TLRs, these receptors dimerize and undergo conformational alterations required for the recruitment of adaptor proteins containing a TIR domain, including myeloid differentiation factor 88 (MyD88), to the cytoplasmic portion of the TLRs by the interaction of their TIR domains. This binding triggers downstream signaling cascades, such as NF-kB pathway, leading to the induction of genes involved in host defense response, including the genes responsible for the production of proinflammatory cytokines, such as IL-1, and chemokines, triggering the inflammatory response (Athman and Philpott 2004, Philpott and Girardin 2004, Akira et al. 2006). The distinct responses mediated by different PAMPs can be explained in part by the selective usage of these adaptor molecules that are responsible for the activation of distinct signalling pathways (Takeda and Akira 2005, Akira et al. 2006).

The recognition of PAMPs by TLRs enables the innate immune system to distinguish which is self from non-self and is important not only for triggering the innate immune response against microbial infection but also for inducing the adaptive immune response (Athman and

Philpott 2004). Thus, TLRs allow the identification of the nature of the pathogen agent and trigger the most appropriate response (Akira et al. 2006).

Toll like receptor system is not able to detect pathogens that invade the cytosol of cells. Thus, these microorganisms are detected by cytoplasmic PRRs, the NLRs. These receptors are cytoplasmic surveillance proteins, detect potentially harmful microorganisms through PAMP recognition and initiate an inflammatory reaction in order to induce the host defense response and fight against the infection (Philpott and Girardin 2004, Akira et al. 2006).

There are two types of NLRs, Nod1 and Nod2, and both recognize bacterial peptidoglycan, although requiring distinct motifs of this molecule to achieve detection. Nod1 recognizes γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and the ligand for Nod2 is muramyl dipeptide (MDP) (Akira et al. 2006, Claes et al. 2014).

Nuleotide-binding oligomerisation domain like receptors contain a C-terminal domain of leucine-rich repeats (LRRs) that recognizes the peptidoglycan, a central nucleotide oligomerization domain (NOD) and a N-terminal in which, Nod1 has one caspase-activating and recruitment domain (CARD) and Nod2 has two of these domains (Akira et al. 2006, Bourhis and Werts 2007).

After the ligand binding, NLRs oligomerize and initiate different signalling cascades. For example, NOD1 and NOD2 can interact with RIP2 kinase by their CARD domains and this serine/threonine kinase function as a potent activator of NF-kB transcription factor, leading to the production of proinflammatory cytokines and chemokines (Akira et al. 2006, Broz et al. 2012).

Ligand recognition by NLRs results not only in the production of cytokines and chemokines, but also in the assembly of inflammasomes, a complex of proteins responsible for the activation of caspase-1 which catalyses the pro- IL-1β processing to produce the mature cytokines (Akira et al. 2006, Wick 2011).

Thus, NODs are very important in mucosal barrier, where most of the membranous TLRs are not expressed and in pathogens that escape from TLR sensing. In contrast to TLRs which recognize bacteria, viruses, fungi and protozoa, NLRs detect bacteria with known functions (Akira et al. 2006, Bourhis and Werts 2007).

Toll like receptors and NLRs are considered pathogen sensors that co-operate in innate immunity. In particular, synergistic crosstalk of Nod2 with TLR2 and/or TLR4 enhances cytokine production and strengthens intestinal barrier function (Creagh and O'Neill 2006, Claes et al. 2014). However, Richardson and their collaborators have already shown an antagonistic role of Nod2 in TLR signaling (Richardson et al. 2010).

2.2 COMPLEMENT

Complement is an enzymatic system of serum proteins that circulate in an inactive state and is composed of nine major components (C1 - C9). A variety of specific and nonspecific immunologic mechanisms can convert these inactive elements into an active state in which a sequential activation of these components (complement cascade) occurs, leading to the damage of pathogen membranes, either by the destruction of the pathogenic organisms or by the facilitation of their clearance (Beutler 2004, Kindt et al. 2007).

Complement components can be sequentially activated through two pathways: (1) the classical pathway and (2) the alternative pathway. Complement is considered as part of the innate immunity because its components play a role in phagocytic chemotaxis, opsonization and the inflammatory response, and may be involved in bacterial killing by the lysis of certain bacteria. However, complement can be triggered by reactions between antibodies (Ab) and antigens (Ag) and, therefore, it may also play a role in adaptive immunity (Male et al. 2006, Kindt et al. 2007).

Complement is activated in the classical pathway by reactions between an Ab of the host and an Ag present on bacteria surface. Some immunoglobulins (IgG and IgM) can bind the complement because they have a complement binding site on its Fc portion. The reaction between IgG and Ag activates the complement in C1 and initiates the cascade reaction on the bacterium surface, resulting in the principal effects of the complement: (Basset et al. 2003, Male et al. 2006, Kindt et al. 2007)

- Generation of inflammatory factors, C3a and C5a, which direct antimicrobial serum factors and leukocytes into the infection site.
- Attraction of phagocytes - Chemotactic factors C3a and C5a attract phagocytes to the infection site.
- Enhancement of phagocytic engulfment - C3b component on Ag - Ab complex attaches to C3b receptors on phagocytes and promotes opsonization of Ab-coated cells. C3b-opsonization is important when Ab is IgM because phagocytes have receptors for IgM only when it is associated with C3b.
- Cell lysis – Five proteins of the complement (C5-C9) generate the membrane attack complex (MAC). When the MAC is formed in plasmatic membrane, it forms a pore in target membrane, allowing the entry of sodium and water into the cell, leading to cell lysis.

- Lysis of bacterial cells mediated by lysozyme – When the MAC is formed in the outer membrane of gram-negative bacteria, the lysozyme enzyme passes through the formed pore and gains access to peptidoglycan, hydrolysing the bacterium cell wall.

In addition to the classical pathway of complement activation, there is an alternative pathway that is independent of immunoglobulins. Activation of this pathway requires the spontaneous activation of C3. Usually, activated C3 is rapidly inactivated by surface proteins present in organism cells. However if activated C3 binds to a pathogen, this element can become stable and activate the complement cascade, leading to the assembly of the terminal attack complex on the organism surface, killing it. The presence of C3b on the microorganism surface can act as an opsonin, increasing the uptake of pathogens by phagocytes through their C3b receptors and resulting in rapid clearance of the organism from the body (Basset et al. 2003).

Finally, there is another complement pathway, the lectin pathway that is similar to the classical pathway. However, in contrast to classical complement pathway, it does not recognize an antibody bound to its target. The activation of this pathway is dependent on the binding of a lectin to a mannose (Mannan-binding lectin - MBL), glucose or others sugars with 3 or 4 OH groups placed in terminal positions on glycoprotein components of microorganisms, such as, *Listeria* or *Salmonella* (Wallis et al. 2010).

2.3 INFLAMMATORY RESPONSE

When the outer barriers of innate immunity, skin and mucosa, are damaged, the innate responses to infection can induce a cascade of events known as inflammatory response. Inflammation is one of the most important responses of the innate immune system in order to fight the bacterial infection, being required for the proper functioning of host defenses. During inflammation, antimicrobial factors, including phagocytes, lymphocytes, antibodies, complement and other antimicrobial plasma molecules are attracted to the infection site. Pro-inflammatory cytokines are also released (Kindt et al. 2007, Broz et al. 2012).

Additionatly, the inflammation is characterized by the increase of blood flow and temperature in tissues, which favours maximal metabolic activity of leukocytes. On the other hand, the inflammatory response increases the vascular permeability that allows the entry of fibrinogen and complement from blood to tissues. The fibrinogen is converted into fibrin that isolates the infected area, avoiding the dissemination of infection. Thus, inflammatory process is very important to prevent the spread of infection and to promote pathogen clearance (Basset et al. 2003, Broz et al. 2012).

During a bacterial infection, resident tissue cells, including macrophages, mast and dendritic cells, are activated by pathogen-associated molecular patterns (PAMPs) to release the initial components of cellular innate immune responses, including the proinflammatory cytokines (TNF-α, IL-1, and IL-6) and chemokines (Male et al. 2006, Kindt et al. 2007).

Tumor necrosis factor-α (TNF-α) has many functions in the development of inflammation because it induces the adhesion molecules and chemokines on the endothelium which are pivotal for the accumulation of leukocytes and it activates the microbicidal systems of phagocytes. Additionatly, TNF-α acts on TNF receptor (TNFR), leading to the activation of AP-1 and NF-κB transcription factors, which, in turn, activate many genes involved in innate and adaptive immune responses. Moreover, TNF-α can induce apoptosis in susceptible cells through the activation of caspase 8 (Beutler 2004, Male et al. 2006).

The interleukin 1 (IL-1) and -6 (IL-6) are pro-inflammatory cytokines produced by many types of cells, including monocytes, macrophages, dendritic or epithelial cells. They act locally on blood vessels and in other cells to increase vascular permeability and help to recruit and activate cells at infection sites. Furthermore, these cytokines can also act on bone marrow during haematopoiesis to enhance the production of neutrophils and other myeloid cells which contribute to pathogen clearance (Male et al. 2006, Kindt et al. 2007).

However, some cytokines can also act as negative regulators of immune response. An example of this function is IL-10 which is immunosuppressive in several ways: it inhibits the macrophage activation and the production of reactive oxygen and nitrogen intermediates, suppresses the production of proinflammatory cytokines and down-regulates the production of important molecules in triggering of specific immunity, such as class II major histocompatibility complex (MHC)-antigen-presenting complex. This immunosuppressive cytokine may also contribute to the generation of T suppressor cells that down-regulate the activation of immune response (Male et al. 2006, Kindt et al. 2007, Ryan et al. 2010).

On the other hand, dying cells release cytoplasmic constituents that lower the pH in the surrounding extracellular environment. This increased acidity of extracellular environment activates an extracellular enzyme, the kallikrein, which in turn activates bradykinin. This peptide binds to receptors on the capillary walls opening junctions between cells to allow leakage of plasma components, which forms the inflammatory exudate (Male et al. 2006, Kindt et al. 2007, Bjorkqvist et al. 2013).

The bradykinin is responsible for the increase of capillary permeability that allows the migration of leukocytes from vessels to tissues, a process called diapedesis. The first cells to appear, and the most dominant, are neutrophils, which are actively phagocytic and characteristic of the acute inflammation. On the other hand, the chronic inflammation has a

higher proportion of macrophages, cytotoxic T cells and even B cells in infection sites (Male et al. 2006, Kindt et al. 2007, Bjorkqvist et al. 2013).

To conclude, it is important to mention that inflammation is also an important aspect of bacterial pathogenesis since the inflammatory response induced by a bacterium can result in considerable damage to the host and, therefore, be part of the pathology of microbial disease (Ryan et al. 2010).

2.4 PHAGOCYTOSIS

When a pathogen invades the host tissues, the inflammatory response is immediately triggered with the recruitment of phagocytes to the infection site. Then, the phagocytosis is a defense mechanism based on the ingestion and destruction of microorganisms (Kindt et al. 2007).

The delivery of phagocytic cells, neutrophils and monocytes, to the site of bacterial infection is possible by three simple steps (Kindt et al. 2007):

1) **Margination** - the adherence of cells to the endothelial wall of the blood vessels;
2) **Diapedesis** – the migration of cells across vascular walls;
3) **Chemotaxis** - the migration of leukocytes to the invasion site in response to chemical signals, including bacterial products, cell and tissue debris and components of the inflammatory exudate.

During an acute inflammation, neutrophils are rapidly recruited from blood to the infection site by the cytokine IL-6 and other factors, being the first cells to accumulate around the invaders and initiate the phagocytic process. After engulfment of bacteria, neutrophils produce nitrogen and oxygen reactive species that can kill bacteria. Furthermore, neutrophils secrete chemokines such as CSF-1 and MCP-1, which attract macrophages to the infection site (Zenewicz and Shen 2007, Sotolongo et al. 2012).

Macrophages consist in monocytes that left the blood and entered in tissues. These cells arrive later to the infection site and are mainly involved in chronic infections. In response to infection, macrophages secrete TNFα and IL-12, two cytokines that drive NK cells to produce interferon gamma (IFN-γ) which, in turn, leads to the activation of macrophages and increases their bactericidal activity (Zenewicz and Shen 2007, Sotolongo et al. 2012).

On the other hand, the macrophages have another indispensable function in host defense: these cells "process" the antigenic components of infective agents and present them to lymphocytes, a mechanism that is usually required for the initiation of adaptive immune responses in host. Thus, the macrophages and related dendritic cells are antigen-presenting

cells that link the innate and adaptive immune system by producing cytokines that enhance innate immune cell function and contribute to lymphocyte function (Greenberg and Grinstein 2002, Male et al. 2006)

The delivery of phagocytic cells to the infection site is followed by the phagocytic adherence to the target. This step involves several types of receptors present on the phagocyte membrane that detect the presence of invading microbes and bind opsonized microbial surfaces. Fc receptors or complement receptors are examples of receptors that may cooperate to determine the cellular response (Greenberg and Grinstein 2002, Kindt et al. 2007).

The presence of opsonins in microbes' surface, such as the complement C3b, increases the rate of adherence and ingestion of the pathogen. The adherence of a phagocyte to a particle is weaker in the absence of opsonization because the adherence occurs by net surface charge on the phagocyte or hydrophobicity of the particle (Beutler 2004, Kindt et al. 2007).

The attachment of the phagocyte to its target particle leads to the ingestion of the particle by endocytosis, resulting in formation of the phagosome, a membranous vesicle derived from plasma membrane. This vesicle migrates into the cytoplasm and merges with digestive granules that discharge their microbicidal contents, such as lysozyme, proteases or hydrolases, into the phagosome, forming a digestive vacuole, the phagolysossome, where the bacteria are killed and degraded to low molecular-weight components (Male et al. 2006, Kindt et al. 2007).

These activities differ in neutrophils and macrophages. On the one hand, neutrophils die and lyse after extended phagocytosis, death and digestion of bacterial cells. On the other hand, macrophages release digested debris and process antigenic components of bacteria that insert into plasma membrane and associate with class II MHC for antigen presentation to T cells during adaptive immune response (Male et al. 2006, Kindt et al. 2007).

3. ADAPTIVE IMMUNE RESPONSE

The innate immune system is very important for the early control of bacterial replication and successful eradication of an infection. However, in vertebrates the innate immune response does not operate alone, since the adaptive immune system allows the specific immune response against a particular microorganism, helping in the clearance of the infection (Hornef et al. 2002, Philpott and Girardin 2004). Furthermore, the adaptive immunity has a memory component that, through memory cells, allows a much faster response and with

greater efficiency to a subsequent infection with the same pathogen (Philpott and Girardin 2004, Kindt et al. 2007).

The innate and adaptive immunity function as a highly interactive and cooperative system, generating a combined response more effective than each could produce by itself to protect the body against foreign invaders. However, for innate and adaptive immunity to work together, these systems must communicate with each other. This communication is performed by cell-cell contact, since the adaptive immune system requires the cooperation between lymphocytes and antigen-presenting cells, so that the lymphocytes may recognize the antigen that activates them. The connection between innate and adaptive immune system can also occur by soluble messengers, such as cytokines, that modulate the function of other cells by binding to specific receptors (Kindt et al. 2007).

Typically, there is an adaptive immune response against a pathogen within 5 or 6 days after the initial exposure, followed by a gradual resolution of the infection. Unlike innate immune responses, adaptive immune responses are not the same in all mammalian species but are reactions to specific antigenic challenges (Chaplin 2006, Kindt et al. 2007).

Adaptive immune responses are carried out by the lymphocytes and may be divided in two main groups: cell-mediated immunity, under the control of T lymphocytes and humoral immunity mediated by B lymphocytes. Lymphocytes are one of many types of leucocytes produced in the bone marrow by the process of haematopoiesis and are essential to an effective immune response (Kindt et al. 2007, Alberts et al. 2008).

3.1 CELL-MEDIATED IMMUNITY

In cell-mediated immune response, T lymphocytes are developed in thymus from common lymphoid progenitors that come from the bone marrow. These cells express a unique antigen-binding molecule, the T cell receptor (TCR) on its membrane. Unlike membrane-bound antibodies on B cells, which can recognize antigens alone, TCR recognize only antigens that are bound to cell-membrane glycoproteins, the major histocompatibility complex (MHC). This condition ensures a highly regulated activation of T cells, which is crucial for immune response, since an inappropriate T-cell response to self-components can result in auto-immune consequences (Akira et al. 2006, Male et al. 2006, Kindt et al. 2007, Bonilla and Oettgen 2010).

There are two major types of MHC molecules: Class I and II. Class I MHC molecules are found on nucleated cells of vertebrate species and their function is to express the antigens produced within the cell on the cell surface. On the other hand, class II MHC molecules which

are expressed only by APCs, such as macrophages or DCs (Kindt et al. 2007, Bonilla and Oettgen 2010).

T cells comprise two major subpopulations: T helper cells (T_H or $CD4^+$) that only recognize antigens associated with class II MHC molecules and T cytotoxic cells (T_c or $CD8^+$) which recognize only antigens associated with class I MHC molecules (Kindt et al. 2007).

T helper cells can also be subdivided in two categories of cells: 1) T helper 1 (T_{H1}) cells that interact with mononuclear phagocytes and help them to destroy intracellular pathogens by IFN-γ production; 2) T helper 2 (T_{H2}) cells which interact with B cells and help them to divide, differentiate, and make antibody (Male et al. 2006). Activated DCs express costimulatory molecules essential to T_H cell activation and that can instruct the differentiation of CD^{4+} cells into T_{H1} or T_{H2} cells (Akira et al. 2006).

In turn, cytotoxic T lymphocytes are responsible for the destruction of host cells that are infected by viruses or other intracellular pathogens (Male et al. 2006).

After a T_H cell recognize and interact with an antigen–Class II MHC molecule, the T_H cell is activated, becomes an effector cell and secretes various cytokines which activate other cells, including B cells, T_c cells, macrophages and other cells that participate in the immune response. Differences in the pattern of cytokines result in different types of immune response and the activation of both, humoral and cell-mediated immunity, requires cytokines (Kindt et al. 2007).

Under the influence of T_H-derived cytokines, a T_C cell that recognizes an antigen–Clss I MHC molecule proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL) which mediate the death of altered self-cells (Kindt et al. 2007).

3.2 HUMORAL IMMUNITY

In the adaptive humoral immunity, B-lymphocytes arise from hematopoietic stem cells in bone marrow and express antigen-binding receptors on their membranes, the IgM antibody molecule. Antibodies are glycoproteins produced by plasma cells and are found in the blood and tissue fluids. These glycoproteins consist of two identical heavy polypeptide chains and two identical light polypeptide chains. The amino-terminal ends of the pairs of heavy and light chains form a cleft within which antigen binds (Male et al. 2006, Bonilla and Oettgen 2010).

When a naive B cell (B cell prior to antigen binding) meets the antigen that matches with its membrane bound antibody, binds to it, leading to rapid cell division and its progeny differentiates into memory B cells and effector B cells. These latter cells produce antibodies in a form that can be secreted (Kindt et al. 2007).

The secreted antibodies are the major effector molecules of humoral immunity, since they bind to the antigens, neutralize and inactivate microbial toxins by coating them and, subsequently, block their binding capacity to the host cells receptors. Antibody binding also marks invading pathogens for destruction, either by making easier for phagocytic cells of the innate immune system to ingest them or by the activation of the complement system, resulting in the lysis of foreign organisms (Kindt et al. 2007, Alberts et al. 2008).

In summary, the activation of the adaptive immune response occurs through cytokine secretion particularly by effector T_H cells and through the antigen recognition by B cells antibodies or T - cell receptors. Furthermore, the binding of Class I and Class II MHC molecules to the antigen, antigenic processing and presentation as well as proliferation and differentiation of effector cells also have an important contribution to control bacterial infections in adaptive immunity (Kindt et al. 2007).

4. BACTERIAL STRATEGIES AGAINST HOST IMMUNE RESPONSES

The success of many pathogens relies on the development of a variety of mechanisms to circumvent, resist or counteract the host immune responses that would eliminate them. In some situations, the pathogens can benefit from the stimulation of host innate responses, since the activation of the immune system can lead to the disruption of the epithelial barrier, thereby facilitating the bacterial invasion (Hornef et al. 2002).

Additionatly, bacteria adopt strategies to escape from the host in different stages: during immune recognition by mucosal surfaces and TLRs; inflammatory response or chemotaxis; recognition and death by phagocytes or during adaptive defense mechanisms.

Bacterial pathogens must at first disrupt physical barriers, such as the mucous membranes (Coombes et al. 2004). Some pathogens are able to avoid the immune recognition by mucosal surfaces through the prevention of opsonization and complement activation, since the proteolytic degradation of secretory immunoglobulin is promoted. Furthermore, the secretion of bacterial toxins can block the host defenses and disrupt its mucosal integrity, thereby facilitating the colonization of host surfaces (Hornef et al. 2002).

Some intracellular parasites, such as *L. monocytogenes* and *S.* Typhimurium, have complex machineries for cellular invasion. These systems involve various types of non-toxin virulence factors, such as adhesins and invasins, which are essential for pathogens to approach cellular surfaces and avoid mechanical removal, helping bacteria to invade the host and to spread within their tissues (Hornef et al. 2002, Coombes et al. 2004, Akira et al. 2006, Male et al. 2006).

Bacteria are also able to exploit the TLR system to evade host immune responses. Pathogens can avoid innate recognition through steric shielding or modification of exposed PAMPs, preventing its binding to PRRs (Akira et al. 2006). Bacterial capsular structures have this role and have long been recognized as important virulence factors (Hornef et al. 2002, Coombes et al. 2004). Alternatively, since the expression of recognition receptors seems to be organ-specific, recognition might be avoided through the selection of certain favourable anatomical sites for colonization and invasion (Hornef et al. 2002).

On the other hand, some pathogens are able to avoid provoke a wide inflammatory response or inhibit phagocytes chemotaxis. Upon arrival at the sub-epithelial space, pathogens find locally phagocytic cells that are attracted to the infection site. Some bacteria can cover their surface with a component which is seen as “self” by the host phagocytes and immune system and thus, these bacteria are able to hide its antigenic surface (Coombes et al. 2004). Bacteria can also avoid the contact with phagocytes by remaining confined in regions that are inaccessible to these cells, such as the lumen of glands in internal tissues or unbroken skin in surface tissues (Hornef et al. 2002, Coombes et al. 2004).

Some pathogenic bacteria employ strategies to avoid the engulfment by phagocytes and are able to resist to the bactericidal components of host tissues due to some structural property. For example, the LPS in the outer membrane of Gram-negative bacteria, such as *Salmonella* Typhimurium, is not easily penetrated by hydrophobic compounds such as bile salts in the GI tract that are harmful to the bacteria and may protect the cells from complement-mediated lysis or the action of lysozyme (Kindt et al. 2007).

Additionally, many gram-positive pathogens, including *Listeria monocytogenes*, are able to secrete extracellular substances that kill phagocytes, acting either as enzymes or "pore-formers" that lyse the membrane of these cells. Some of these substances are described as hemolysins or leukocidins due to their lethal actions against red blood cells or leukocytes, respectivetly (Coombes et al. 2004).

Furthermore, some bacteria are able to survive or multiply within the phagocytes by a set of mechanisms that interfere with the bactericidal activities of the host cells. The intracellular environment of a phagocyte may protect the bacteria during the early stages of infection or until they develop a full complement of virulence factors. The intracellular environment also protects the bacteria against the activity of extracellular bactericides, antibodies or drugs (Coombes et al. 2004). Alternatively, bacteria can interfere with endosomal trafficking, persist in modified phagosomes or resist to the death by lisosomal constituints. The mechanisms behind this resistance to phagocytic killing are poorly understood. However, it is speculated that it may be due to bacteria surface components or

extracellular substances that bacteria produce, which interfere with the mechanisms of phagocytic killing (Hornef et al. 2002, Coombes et al. 2004).

Bacteria have also several strategies to escape from the adaptive immune response. Some bacterial pathogens have the ability of modulating cytokine production in the host, including the induction of immunosuppressive cytokines, such as IL-10 and transforming growth factor β (TGF-β), which depress the immune system. On the other hand, bacteria can also inhibit the production of pro-inflammatory cytokines and the surface expression of co-stimulatory molecules such as CD86 by APC (Giacomini et al. 2001, Hornef et al. 2002, Ruckdeschel et al. 2002).

Interfering with antigen processing or class I and class II MHC expression is another strategy to prevent the stimulation of an adaptive immune response, leading to diminished antigen presentation. Alternatively, some bacteria interfere with the capacity of T and B cells to carry out their functions either by inducing suppressor T cells and thus depressing the immune response or by mediating T cell apoptosis by induction of Fas ligand (FasL) expression on T cells (Ullrich et al. 2000, Hornef et al. 2002).

5. IRON, AN ESSENTIAL ELEMENT TO HOST AND PATHOGEN

Iron is the fourth most common element in the Earth's crust and the most abundant transition metal in the human body (Vyoral and Petrak 2005).

This element is essential for both microbial pathogen and mammalian hosts during the course of disease, being considered a key element in host-pathogen interactions (Doherty 2007, Cherayil 2011, Nairz et al. 2014).

Iron is required by nearly all living organisms, which use it as a cofactor or prosthetic group for numerous enzymes, since the redox potential of the Fe^{2+}/Fe^{3+} switch is used in important biological systems of both eukaryotes and prokaryotes (Doherty 2007). These systems include basic metabolic pathways, such as the respiratory pathways, and cellular processes ranging from electron transport, energy generation and DNA replication to oxygen transport in haemoglobin and myoglobin and protection against oxidative stress. This transition metal is also required for various host innate defense mechanisms, including the production of reactive oxygen and nitrogen intermediates. Furthermore, iron has synergistic effects towards anti-microbial radical formation and alters the immune cell proliferation, as well as, the anti-microbial immune effector pathways (Schaible and Kaufmann 2004, Skaar 2010, McLaughlin et al. 2011, Nairz et al. 2014, Lechowicz and Krawczyk-Balska 2015).

However, iron can also be toxic to cells when present at high concentrations due to its ability to promote the formation of damaging oxidative radicals which can intoxicate microbes or damage surrounding cells and tissues (Andrews and Schmidt 2007, Nairz et al. 2014).

Apart from direct effects on innate immunity, iron availability also influences acquired immune responses. Iron deficiency down-regulates T-cell responses in several experimental models, reducing antigenic-specificity as well as polyclonal proliferation. The CD28, an important co-stimulatory receptor that functions in T-cell activation, is down regulated under iron deficiency conditions (Schaible and Kaufmann 2004).

Iron-overloaded mice showed reduced contact-mediated sensitivity reactions as well as lower IFN-γ production and IgM secretion. However, and surprisingly, in iron-deficient mice, a similar outcome was observed (Schaible and Kaufmann 2004).

Since both iron deficiency and iron excess can compromise host immune responses, vertebrate animals strictly regulate iron absorption in order to maintain the normal iron balance since they have no specific mechanism of iron excretion (Vyoral and Petrak 2005, Cherayil 2011). The normal iron balance is crucial for optimal functioning of the host immune system (Schaible and Kaufmann 2004)

5.1 IRON METABOLISM IN THE HOST

Iron is essential for many biologic functions of the host, including oxygen sensing and transport, energy metabolism, antimicrobial defense and erythropoiesis. The latter mechanism is the highest consumer of iron in the mammalian organism, 60–70% of the total iron in the human adult body being bound to the heme subunit of red blood cells (RBCs) haemoglobin (Schaible and Kaufmann 2004, Silva-Gomes et al. 2013).

In the cytoplasm, iron exists predominantly in the ferrous form (Fe^{2+}). However, free ferrous iron is highly toxic to cells, since it can react with H_2O_2 to generate highly toxic hydroxyl radicals via the Fenton reaction, resulting in protein denaturation, DNA breaks and lipid peroxidation (Achard et al. 2013). Thus, the vertebrates have evolved a complex network of proteins to acquire, transport and store iron, preventing its occurrence in a free form. In normal conditions, extracellular iron is kept bound to transferrin and intracellular iron is stored within the ferritin (Schaible and Kaufmann 2004, Hentze et al. 2010, McLaughlin et al. 2011).

In mammals, macrophages of the reticuloendothelial system play a central role in the regulation of iron metabolism since these cells recycle heme iron from senescent RBCs and regulate the storage and release of iron. Phagocytosed erythrocytes are degraded in lysosomes, and iron is released from haem via haem oxygenase 1 (HO-1). Iron is then released

to the cytoplasm of the cell by the divalent metal transporter 1 (DMT1) (Evstatiev and Gasche 2012).

Another iron import pathway includes the recovery of iron complexed to haemoglobin or haptoglobin from the lumen of macrophages by the haemoglobin scavenger receptor (CD163), present in apical membrane of these cells. Some of the iron may be used by the cell for metabolic purposes and the excess is either stored in association with ferritin or pumped out of the cell by the exporter protein, ferroportin. Once outside the cell, Fe^{2+} is oxidized to Fe^{3+}, either by the intestinal membrane-associated ferroxidase, hephaestin, or the plasma-located ferroxidase, ceruloplasmin. In plasma, iron is transported in association with transferrin (Schaible and Kaufmann 2004, Stein et al. 2010, Evstatiev and Gasche 2012, Silva-Gomes et al. 2013).

Cytokines allow an efficient iron storage within macrophages by increasing the levels of the iron storage protein, ferritin (Nairz et al. 2014).

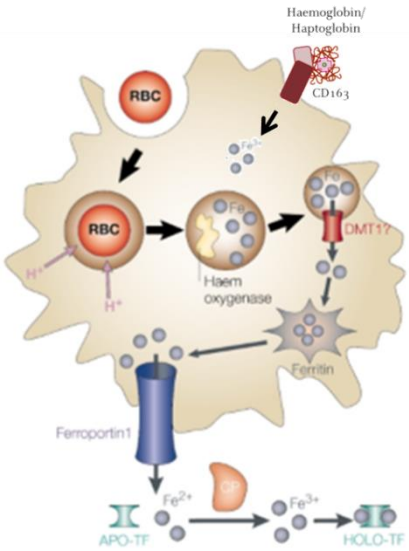

Figure 1: Iron transport in macrophages. Senescent RBCs are phagocytosed by macrophages and Fe^{2+} is released from haem by HO-1. Iron is probably released to the cytoplasm of the cell via DMT1. Iron complexed to haemoglobin or haptoglobin is taken up by macrophages through the haemoglobin - scavenger receptor – CD163. Inside the cell iron is stored in ferritin or exported from the cell by ferroportin. In plasma, Fe^{2+} is converted to Fe^{3+} by ceruloplasmin or by hephaestin. Adapted from (Andrews 2000).

A small amount of iron is also absorbed from the diet. Dietary iron, either free ionic iron or complexed to heme or other chelators, is absorbed at the brush border of enterocytes lining in the proximal portion of the duodenum. In intestinal lumen, free ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by duodenal cytochrome-b-like reductase (DCYTB), located on the apical membrane of the enterocytes, facilitating the absorption of Fe^{2+} from the lumen into

the cytoplasm through DMT1 (Schaible and Kaufmann 2004, Knutson et al. 2005, Stein et al. 2010).

On the other hand, iron complexed to heme crosses the apical membrane of enterocytes likely through the haem carrier protein 1 (HCP1). Inside the cell, heme iron is metabolized by a reaction catalyzed by HO-1 (Wang and Pantopoulos 2011, Evstatiev and Gasche 2012). Iron absorption by enterocytes in the proximal intestine is strictly controlled in order to maintain body iron homeostasis (Stein et al. 2010, Vanoaica et al. 2010, Cherayil 2011).

Inside the enterocytes, some iron is stored within ferritin and the remainder iron is released to the outside of the cell through the basolateral membrane exporter, ferroportin. In plasma, a ferroxidase converts Fe^{2+} to Fe^{3+} which, in turn, binds to transferrin (Cherayil 2011) whose function will be clarified in the next section.

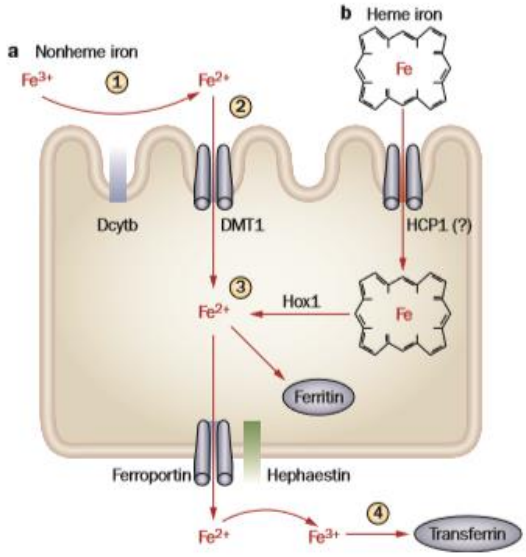

Figure 2: Duodenal absorption of heme and nonheme iron. (1) In intestinal lumen, nonheme Fe^{3+} is reduced to Fe^{2+} by DCYTB. (2) Fe^{2+} is absorbed into the cytoplasm of enterocytes via DMT1. (3) Heme iron crosses the apical membrane of enterocyte membrane likely through HCP1 and is liberated from the porphyrin framework by HO-1. Within the intestinal epithelial cell, iron either binds to ferritin or is exported to the outside of the cell by ferroportin. (4) Hephaestin oxidizes Fe^{2+} to Fe^{3+}, which binds to transferrin (Stein et al. 2010).

5.1.1 TRANSFERRIN, THE PLASMA IRON TRANSPORTER PROTEIN

Transferrin, one of the major serum proteins in eukaryotes and is a powerful chelator that belongs to a family of homologous iron-binding glycoproteins that encompasses lactoferrin, melanotransferrin and ovotransferrin. Transferrin is a single polypeptide chain of

76–81 kDa, comprised by two structurally similar globular domains, N- and C-lobes, each containing a single iron binding site (Gkouvatsos et al. 2012).

Transferrin is mainly synthesized in the liver and secreted into the blood where it is able to bind iron with high affinity although this binding is reversible (Lambert et al. 2005). Transferrin can bind two atoms of Fe^{3+}, which is present at higher concentrations in extracellular areas at pH 7.4 and at lower concentrations in the acidified endosomes, where Fe^{3+} is converted into Fe^{2+} (Gkouvatsos et al. 2012, Silva-Gomes et al. 2013).

Iron chelation by transferrin has three main purposes: i) it maintains Fe^{3+} in a soluble form under physiological conditions; ii) it facilitates iron transport and its cellular uptake, and iii) it maintains Fe^{3+} in a redox-inert state, preventing the generation of toxic free radicals. Thus, transferrin has an indirect role against systemic infections by creating an environment with low levels of iron, where few pathogens can survive and proliferate (Gkouvatsos et al. 2012).

The liver plays a key role in homeostatic maintenance of serum iron levels that involves the sensing of iron-transferrin complexes followed by the activation of regulatory mechanisms that appropriately adjust the amount of iron that enters in the circulation (Vanoaica et al. 2010, Cherayil 2011).

Under normal conditions, most of the iron in the blood is bound to transferrin and when this protein is saturated, iron-transferrin complexes bind with high affinity to the transferrin receptor (TFR), which is expressed on the cell surface and is internalized by clathrin-dependent endocytosis. Next, iron is transferred to the early endosome, where it is exported into the cytoplasm through the DMT1 present in the membrane of the endosome (Schaible and Kaufmann 2004, Knutson et al. 2005, Weiss 2005, Gkouvatsos et al. 2012, Silva-Gomes et al. 2013). The iron excess is stored in the hepatocyte ferritin. When iron is required for cellular metabolism, ferritin releases the metal that is exported into the plasma by a ferroportin-dependent process (Schaible and Kaufmann 2004, Knutson et al. 2005, Weiss 2005, Vanoaica et al. 2010, Wang and Pantopoulos 2011).

Tumor necrosis factor-α, IL-1, IL-6 and IFN-γ increase the uptake of transferrin and non-transferrin bound iron by modulating the expression of TFR-1 and DMT1, respectively (Silva-Gomes et al. 2013, Nairz et al. 2014).

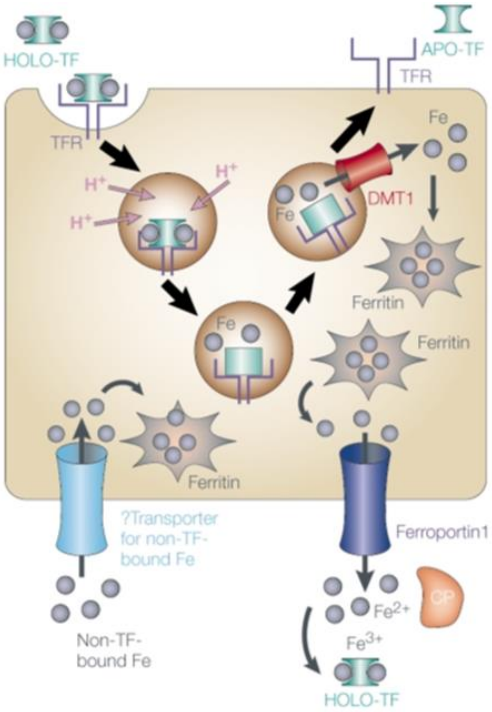

Figure 3: Iron transport in hepatocytes. Iron-saturated transferrin binds to TFR expressed in the cell surface, being endocytosed and delivering iron to the early endosome. Iron is transported into the cytoplasm likely through DMT1 present in the membrane of endosomes. If not immediately used, the iron is stored bound to ferritin. Fe^{2+} is exported from the hepatocytes by ferroportin and is oxidized to Fe^{3+} by hephaestin or ceruloplasmin (Andrews 2000).

5.1.2 FERRITIN, THE CELLULAR IRON STORAGE PROTEIN

Ferritin is a 450 kDa protein polymer of 24 subunits of heavy and light chains with molecular masses of 21 and 19 kDa, respectively. This protein forms a hollow shell structure that can store up to 4500 Fe^{3+} ions in an inert, soluble and non-toxic form. H-ferritin is expressed mainly in the heart and pancreas, it is involved in iron detoxification and it is the only that exhibits ferroxidase activity, a condition to incorporate ferric ion into the ferritin shell, while the L-ferritin is found predominantly in the liver and spleen, being used in iron nucleation, mineralization and long-term storage (Schaible and Kaufmann 2004, Latunde-Dada 2009, Vanoaica et al. 2010)

Ferritin is considered a first defense molecule against invading microorganisms due to its ability to sequester iron from circulation. Decreased serum iron levels are essential during infection and inflammation, since this condition reduces the availability of iron for invading pathogens (Schaible and Kaufmann 2004, Latunde-Dada 2009, Vanoaica et al. 2010, Cherayil 2011).

Acute-phase response to infection by cytokines stimulates ferritin gene expression. Either TNF-α or IL-1, or both, induce H-ferritin expression in mammals and tissues, in order to limit the availability of iron in circulation (Torti and Torti 2002).

5.1.3 FERROPORTIN, THE IRON EXPORTER IN CELL

Iron is exported from cells by a 571 amino acid basolateral permease known as ferroportin, that functions as a transmembrane Fe^{2+} channel allowing iron efflux (Schaible and Kaufmann 2004, Vyoral and Petrak 2005).

The most important cells in the export of iron into circulation are macrophages and enterocytes (Schaible and Kaufmann 2004, Knutson et al. 2005, Weiss 2005, Silva-Gomes et al. 2013).

Ferroportin is the only known iron exporter and is mainly regulated by hepcidin (Schaible and Kaufmann 2004, Vyoral and Petrak 2005, De Domenico et al. 2008, Stein et al. 2010, Singh et al. 2011), which will be discussed in the following section.

5.1.4 HEPCIDIN, A KEY REGULATOR IN IRON METABOLISM

Systemic iron homeostasis is regulated by hepcidin, an antimicrobial, acute-phase peptide with 20–25 amino acid residues, mainly synthesized by hepatocytes in liver and that is considered a key hormone in iron homeostasis in the body (Schaible and Kaufmann 2004, Nairz et al. 2007, Nairz et al. 2008, Singh et al. 2011, Nairz et al. 2014).

Hepcidin regulates the transmembrane iron transport, since it binds to the ferroportin present on basolateral membrane of enterocytes, reticuloendothelial macrophages and hepatocytes, promoting its internalization by endocytosis and lysosomal degradation and leading to the reduction of iron levels in plasma (Vyoral and Petrak 2005, Singh et al. 2011).

Hepcidin expression in mainly regulated at the transcriptional level, depending on several signaling pathways (Stein et al. 2010, Ganz and Nemeth 2012). Hepcidin gene expression is regulated by iron overload, inflammatory stimuli, hypoxia, anaemia or erythroid activity (Viatte and Vaulont 2009).

The iron sensing function is thought to be carried out by the human hemochromatosis protein (HFE), a hepatocyte surface protein, which associates with TFR-1. When serum iron levels and saturation transferrin are high, the iron-transferrin binds to TFR-1 and displaces HFE, which then binds to the related hepatocyte-specific protein, TFR-2. The interaction between HFE and TFR2 activates signaling pathways that promotes the expression of *Hamp*, the gene

that encodes hepcidin (Figure 4)(Schaible and Kaufmann 2004, Knutson et al. 2005, Weiss 2005, Viatte and Vaulont 2009, Ganz and Nemeth 2012).

Furthermore, it is believed that iron absorption in enterocytes leads to the activation of an iron-sensing protein, the bone morphogenetic protein 6 (BMP6), which is delivered to the liver. In this organ, BMP6 binds to its receptor and to the co-receptor hemojuvelin (HJV), activating the *Hamp* promoter by a SMAD-dependent process which results in the synthesis of hepcidin (Stein et al. 2010).

On the other hand, during infection and inflammation, released pro-inflammatory cytokines, such as IL-6, induce hepcidin expression via JAK-STAT signaling pathway (Stein et al. 2010). Hepcidin binds to ferroportin and induces its internalization and lysosomal degradation, thereby decreasing iron export into plasma from duodenal enterocytes, reticulo-endothelial macrophages and hepatocytes (Figure 4) (Vyoral and Petrak 2005, Vanoaica et al. 2010, Cherayil 2011, Ganz 2011, Singh et al. 2011).

In contrast, physiological mechanisms, such as erythropoiesis, act as negative regulators of hepcidin, since iron is essential for the formation and maturation of red blood cells. It has been proposed that an “erythroid regulator” is involved in erythroid suppression of *Hamp* synthesis (Rishi et al. 2015). The erythropoietin (EPO) is one of the main signalling molecules which mediate erythropoiesis and is produced by the kidney when oxygen levels are low. Some studies showed that EPO can supress *Hamp* expression (Ashby et al. 2010, Sasaki et al. 2012). However, these results also suggest that EPO does not act directly on *Hamp* and therefore cannot be the erythroid regulator. It was hypothesized that erythroid factor is a molecule secreted by erythroblasts. Some studies identified potential erythroid regulators, including growth differentiation factor 15 (GDF15)(Tanno et al. 2007), twisted gastrulation BMP signalling modulator 1 (TWSG1)(Tanno et al. 2009) and erythroferrone (ERFE) (Kautz et al. 2014).

The main function of the iron in haemoglobin is bind to the oxygen. During hypoxic conditions, an increase of erythropoiesis occurs via EPO, hence enhancing the oxygen availability. Similar to erythropoiesis, *Hamp* expression decreases during hypoxia. The main mediator of the hypoxic regulation of genes is the transcription factor hypoxia-inducible factor (HIF). This transcription factor can directly bind to hypoxia-responsive elements (HREs) in the *Hamp* promoter and reduces the hepcidin expression (Rishi et al. 2015).

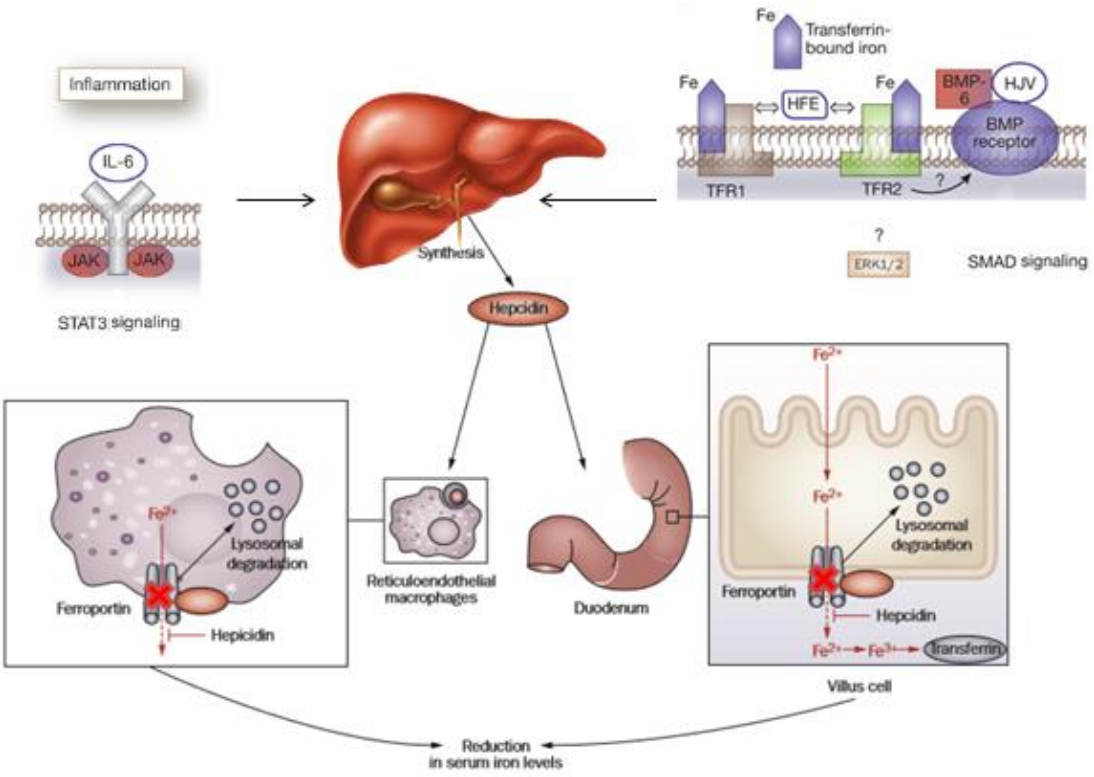

Figure 4: Role of hepcidin in iron metabolism. Hepcidin expression in mainly regulated at transcriptional level by several signaling pathways. Hepcidin gene expression is upregulated during infection and inflammation by IL-6 and when iron levels in plasma are elevated. BMP and HFE regulate hepcidin by sensing enteric iron status. Hepcidin is synthetized by the liver and binds to ferroportin, triggering its internalization and degradation which leads to a reduction in iron release from enterocytes and macrophages to the plasma. Adapted from (Stein et al. 2010).

5.1.5 REGULATION OF IRON AT CELLULAR LEVEL

Intracellular free iron concentrations are influenced by systemic levels of the element, but are also regulated by alterations in the expression of proteins that transport or store iron. The expression of TFR-1, DMT1, ferroportin and ferritin is modulated in response to changes in iron response elements (IREs) present in the 5'or 3'untranslated regions of the corresponding mRNAs. Iron regulatory proteins (IRPs) present in the cytosol bind IREs when intracellular free iron levels are low. The binding of IRPs to the IREs in the 3'untranslated regions of the TFR-1 and DMT1 mRNAs allows the stabilization of the transcripts and increases the expression of the proteins, thus facilitating influx of iron (Andrews and Schmidt 2007, Cherayil 2011, Anderson et al. 2012).

On the other hand, the binding of IRPs to the IREs in the 5' untranslated regions of ferritin and ferroportin mRNAs inhibits their translation, decreasing expression of the proteins,

and preventing storage and export of iron, respectively (Andrews and Schmidt 2007, Cherayil 2011, Anderson et al. 2012).

When intracellular iron levels are elevated, IRPs do not bind IREs, resulting in increased ferritin and ferroportin expression, as well as, TFR-1 and DMT1 degradation (Anderson et al. 2012).

5.2 IRON METABOLISM IN THE PATHOGEN

Upon entering into the host cell, the pathogen find metals in their surroundings and incorporates them in metalloproteins strictly required for its survival. Indeed, iron is a vital nutrient to bacteria, since without this element they are unable to replicate, proliferate and cause disease (Skaar 2010, Silva-Gomes et al. 2013)

Iron is an important growth factor for pathogenic bacteria and high levels of iron are required for various metabolic processes that are crucial for microbial replication, including electron transport, glycolysis, DNA synthesis and defense against toxic reactive oxygen (ROI) and nitrogen (RNI) intermediates (Schaible and Kaufmann 2004, Silva-Gomes et al. 2013).

5.3 THE BATTLE FOR IRON BETWEEN VERTEBRATE HOSTS AND BACTERIAL PATHOGENS

Successful colonization of a host by pathogens requires that these have access to sufficient amounts of iron. However, in response to infection, vertebrate hosts explore this requirement and develop strategies to limit the availability of iron to the pathogen (Silva-Gomes et al. 2013)

One of the most studied strategies employed by the host to reduce the proliferation of the pathogen is the withholding of nutrients in a process termed nutritional immunity. The most significant form of nutritional immunity is the metal deprivation by pumping out the metal from the phagosome, a process mediated by metal transporters such as Nramp1. The metal can then be stored or exported (Skaar 2010, Silva-Gomes et al. 2013).

Additionally, the aerobic environment and neutral pH of serum ensures that extracellular iron (Fe^{3+}) is insoluble which makes it difficult to access by invading pathogens. This difficulty is enhanced by transferrin which binds any extracellular free iron (Skaar 2010). The hypoferremia represents the major host defense strategy and can be also induced through iron sequestration in reticuloendothelial macrophages (Silva-Gomes et al. 2013).

For intracellular pathogens, iron is also restricted thought its sequestration by ferritin or active efflux from the cell by the ferroportin (Crouch et al. 2008).

On the other hand, the host can explore the toxicity of iron and use it on the microbial invader by increasing its concentration in the compartment where pathogens proliferate. By this mechanism, iron can induce pathogen death together with the respiratory burst in phagocytes. The superoxide radical is quickly reduced to hydrogen peroxide which, in turn, reacts with a reduced transition metal, such as Fe^{2+} by the Fenton reaction and gives rise to highly reactive hydroxyl radicals, resulting in oxidative damage to the pathogen (Silva-Gomes et al. 2013).

However, as iron is also a vital element for pathogens, bacteria developed specialized iron-uptake systems that enable them to compete with mammalian hosts for this metal. These systems consist of dedicated transporters and specialized iron binding molecules that function in a highly interrelated mode and include siderophores or heme-sensing systems (Skaar 2010). Iron-uptake systems for specific bacteria will be discussed later.

On the other hand, several pathogenic intracellular bacteria are able to exploit distinct host-cell compartments, using them as niches for survival. These strategies are an important driving force in their growth and survival (Schaible and Kaufmann 2004, McLaughlin et al. 2011).

Iron homeostasis in most bacteria, including *L. monocytogenes* and *S.* Typhimurium, is controlled by the regulatory protein Fur (ferric uptake regulator) or a functional equivalent. In the presence of sufficient levels of iron, Fur acts as a repressor, since Fur–iron complex prevents gene transcription by binding to a specific Fur-box sequence in the promoters of genes encoding for proteins involved in iron utilization. In the absence of iron, Fur-mediated repression does not occur and the genes are transcribed to increase iron levels (Skaar 2010, McLaughlin et al. 2011).

In summary, the modulation of hepcidin, ferroportin, ferritin and transferrin expression during infection and inflammation couples iron metabolism to host defense and decreases iron availability to invading pathogens (Ganz 2011).

6. *LISTERIA MONOCYTOGENES*

The genus *Listeria* consists of a group of gram-positive bacterial pathogens closely related to Bacillus, Clostridium, Enterococcus, Streptococcus, and Staphylococcus. *Listeria* spp. are facultative anaerobic rods of 0.4 by 1 to 1.5 mm that do not form spores and have no capsule. This specie is also catalase-positive, nonacid fast and motile at 10 to 25°C. This motility is produced by approximately 6 flagella (Vazquez-Boland et al. 2001).

Listeria monocytogenes causes serious localized and generalized infections, listerioses, in humans and a variety of other vertebrates (McLaughlin et al. 2011). Listeriosis is an infection usually caused by ingestion of contaminated food with a mortality rate of about 30% (Pamer 2004, Berche 2005). This infection affects individuals with a weakened immune system such as elderly people, pregnant women, newborns or immunocompromised adults (Lecuit 2005, Ramaswamy et al. 2007).

Listeria monocytogenes is a facultative intracellular bacterium that after ingestion can cross the mucosa of gut and disseminate through bloodstream, reaching the central nervous system or the placenta (Berche 2005). This bacterium has the unusual capacity to enter, survive and multiply in both phagocytic and non-phagocytic cells and spread from cell to cell using an actin-based motility process. These properties are considered central for the pathophysiology of human listeriosis (Lecuit 2005, Ramaswamy et al. 2007). The majority of gram-positive bacteria, including *L. monocytogenes*, have a cytoplasmic membrane, surrounded by an assembly of the macromolecular polymer peptidoglycan, but they are devoid of outer membrane. Therefore, the cell surface of *L. monocytogenes* consists of thick sheaths of PG biopolymer to which proteins and other molecules, like techoic acid, covalently or non-covalently associate (Klebba et al. 2012).

The route of infection with *L. monocytogenes* is the gastrointestinal (GI) tract. This pathogen infects intestinal epithelial cells through Internalin A (InlA), a cell surface protein that mediates the attachment to epithelial cadherin (E-cadherin), which is expressed at the surface of host epithelial cells. In the liver, *L. monocytogenes* enters the hepatocytes by another surface protein, internalin B (InlB), which binds to the growth factor receptor tyrosine kinase c-Met present at the cell surface of hepatocyte (Pamer 2004, Lechowicz and Krawczyk-Balska 2015).

6.1 INNATE RESPONSES TO *LISTERIA MONOCYTOGENES*

After infection with *L. monocytogenes*, innate immune responses are rapidly triggered, being essential for early control of growth and dissemination of *L. monocytogenes* and, subsequently, for host survival (Pamer 2004). *L. monocytogenes* expresses several PAMPs, including peptidoglycan (recognised by TLR2) and flagellin (recognised by TLR5). Binding of a PAMP to its TLR initiates a signaling cascade that results in the activation of NF-kB transcription factor, leading to the expression of different genes related with cytokines and antigen production (Pamer 2004, Zenewicz and Shen 2007).

Macrophages are the most important cells of innate immunity during infection with *L. monocytogenes* since bacterial replication occurs primarily within these cells and they have also an essential function in the clearance of bacteria. Resident macrophages, especially hepatic kupffer cells, are responsible for the initial death of the majority of bacteria, partly due to the production of nitric oxide (NO) (Goldfine and Shen 2007, Zenewicz and Shen 2007).

Interferon-γ (IFN-γ) is considered the most important cytokine for controlling a primary *L. monocytogenes* infection due to the resulting activation of macrophages. NK cells are important sources of this cytokine (Pamer 2004, Zenewicz and Shen 2007).

6.2 STRATEGIES OF *L. MONOCYTOGENES* TO ESCAPE FROM THE HOST IMMUNE SYSTEM

L. monocytogenes is able to survive, persist and proliferate in host phagocytes. To avoid the degradation in the phagolysosome, this bacterium relies on several molecules for early lysis of the phagosome to ensure their release into the cytoplasm. These molecules include a bacterial toxin, the listeriolysin O (LLO), and two forms of phospholipase C (PlcA and PlcB) that disrupt the endosomal membrane (Hornef et al. 2002, Coombes et al. 2004, Pamer 2004, Lechowicz and Krawczyk-Balska 2015).

Additionally, the actin-assembly-inducing protein (ActA) is a bacterial protein that nucleates actin, creates actin polymers that promote the bacterial escape from the phagosome, the movement of bacterium in host cytosol and the spreading to neighbouring cells (Pamer 2004, McLaughlin et al. 2011, Travier and Lecuit 2014).

Furthermore, during innate immune responses, *L. monocytogenes* is able to induce type I interferons which in viral infections usually protect the mammalian host. However during bacterial infections, it seems to be beneficial for pathogen either by directly enhancing its growth, or more likely, by downmodulating the host immune responses. These interferons induce T cell apoptosis during *L. monocytogenes* infection, resulting in greater IL-10 secretion by phagocytic cells. Type I IFNs also induces the loss of TNF-producing cells and decreases the viability of macrophages infected with *L. monocytogenes*, leading to decreased bacterial clearance (Pamer 2004, Zenewicz and Shen 2007).

Listeria monocytogenes activates the NF-κB transcription factor as a potential means of increasing its pathogenicity. *L. monocytogenes*-mediated NF-κB activation in endothelial cells results in increased expression of the intercellular adhesion molecule-1 (ICAM-1) and E-selectin and in secretion of IL-8 and macrophage chemoattractant protein-1 (MCP-1). This scenario promotes the attraction of circulating phagocytes and promotes diapedesis, directing

the phagocytes infected with *L. monocytogenes* to the subendothelial space facilitating tissue infiltration and bacterial dissemination (Hornef et al. 2002).

6.3 IRON AND *LISTERIA MONOCYTOGENES*

The direct correlation between iron availability inside the host and the onset and progress of *Listeria* infections has been well documented over the past 30 years (McLaughlin et al. 2011). This correlation was identified because some diseases characterized by iron overload, such as hemochromatosis and thalassemia, present increased susceptibility to the infection with *L. monocytogenes*. Moreover, this pathogen has increased virulence in mice with higher levels of iron (Brown and Holden 2002, Cassat and Skaar 2013).

The ability of this bacterium to acquire and use iron is not only essential during infection but can also support its growth and survival in many diverse environmental niches (McLaughlin et al. 2011). Additionatly, high iron concentrations results in the up regulation of internalin proteins (InlA and InlB) required for bacterial invasion within the host (McLaughlin et al. 2011).

6.3.1 COMPETITION FOR IRON BETWEEN *L. MONOCYTOGENES* AND HOST

As iron is a vital resource for *L. monocytogenes*, host iron sequestration provides a significant barrier to bacterial infection. The competition between host and pathogen for iron forced *L. monocytogenes* to develop counterstrategies to overcome iron-dependent host immune responses. This process is coordinated, at least partly, by the Fur protein, previously mentioned. Disrupting Fur in *L. monocytogenes* results in a significant reduction of its virulence potential (McLaughlin et al. 2011).

A previous study using DNA microarrays (Ledala et al. 2010) identified 14 Fur-regulated genes in *L. monocytogenes*, including genes encoding ferrous iron transporters, ferrichrome ABC transporters, and proteins involved in iron storage (McLaughlin et al. 2011).

6.3.2 IRON ACQUISITION BY *L. MONOCYTOGENES* DURING INFECTION

Listeria monocytogenes has the ability to transport iron, either in the form of ferric siderophores or by extracting it from mammalian iron binding proteins. Despite not producing siderophores, the bacteria can use heterologous siderophores, providing a mechanism for acquiring iron in microbial environment. However, it is unlikely that heterologous siderophores are present in significant quantities within the host and, therefore, alternative iron-uptake mechanisms are required to acquire iron from the host (Brown and Holden 2002).

Listeria monocytogenes mediates the acquisition of several different forms of iron through four distinct mechanisms (Figure 5)(Brown and Holden 2002, Jin et al. 2006, McLaughlin et al. 2011, Klebba et al. 2012, Lechowicz and Krawczyk-Balska 2015):

i. Acquisition of iron from host binding proteins, such as haemoglobin or transferrin. *L. monocytogenes* expresses haemolysin, a protein that is able to lyse erythrocytes and access haemoglobin. In the *L. monocytogenes* genome, the operon hupDGC contains genes encoding for an ABC transporter, which allows the uptake of iron from haemoglobin. However, in *L. monocytogenes* the process of haem acquisition can occur in two ways, depending on the haem concentration in the environment. When the environmental haem concentration is high, free haem molecules are bound by protein HupD anchored to the cytoplasmic membrane and transported into the cell in a process driven by ATP hydrolysis. In contrast, when environmental haem concentration is low, additional surface proteins, namely haem binding proteins (Hbp) 1 and 2, bind haem. While heme can be an essential source of iron for *L. monocytogenes*, its acquisition must be tightly regulated, since high intracellular concentrations of heme can be toxic. In 2010, Anzaldi and their co-workers observed that the genome of *L. monocytogenes* encodes heme-regulated transporter (Hrt) homologs, an efflux system which reduces the toxic effects.

   In relation to transferrin, the transport system responsible for the iron acquisition has not yet been identified.

ii. Extracellular or surface-bound iron reductases allow the capture of soluble ferrous iron, instead of ferric iron. The Feo transport system seems be responsible for Fe^{2+} transport in cytosol of bacterium.

iii. Citrate uptake system, in which the citrate is a ligand for iron and is recognized and bound by a receptor on the surface of bacterial cells.

iv. Ferric siderophore and siderophore-like uptake systems secreted by other microorganisms, since genes responsible for the biosynthesis of siderophores are absent in the genome of *L. monocytogenes*. In the cytosol of bacterial cells, iron-siderophore complex is dissociated to release the iron that can be used in metabolic processes. Dissociation of iron from siderophores is probably due to its reduction to ferrous form that binds to siderophores with relatively low affinity. *L. monocytogenes* is also able to acquire iron associated with hydroxamate siderophores such as ferrichrome. In the *L. monocytogenes* genome, the operon FhuBCDG contains genes encoding for an ABC transporter responsible for the

transfer of ferric hydroxamate siderophores from the environment into the cytosol of bacterium.

The expression of HupDGC and FhuBCDG operon is regulated by Fur protein (Jin et al. 2006, McLaughlin et al. 2011, Lechowicz and Krawczyk-Balska 2015).

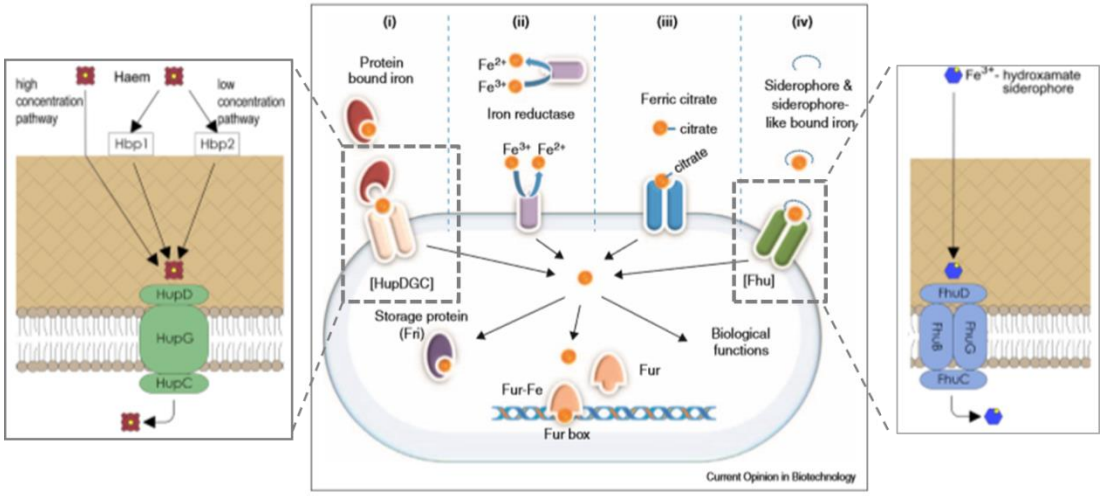

Figure 5: Mechanisms adopted by *Listeria monocytogenes* to obtain iron during infection. *Listeria monocytogenes* have four mechanisms to acquire iron during infection: (i) acquisition of host protein-bound iron by HupDGC, (ii) extracellular and/or surface-bound iron reductases, (iii) ferric citrate uptake system, and (iv) siderophore and siderophore-like uptake systems by Fhu. Within the cell, iron is stored in Fri protein or is used for many biological functions. The Fur regulator binds to the Fur box in the presence of the complex Fur-Fe and is released in low iron conditions. Adapted from (McLaughlin et al. 2011, Lechowicz and Krawczyk-Balska 2015)

6.3.3 STORAGE OF IRON IN *L. MONOCYTOGENES*

The storage of iron within the cell also influences the host-pathogen interactions. Similar to the host, bacteria use ferritin-like proteins for the storage of this metal. *L. monocytogenes* genome has one ferritin encoding gene, designated Fri, which is considered necessary for full *L. monocytogenes* infection, since iron can be stored bound to it (Figure 5) (Ledala et al. 2010, McLaughlin et al. 2011).

Fri deletion leads to an increased sensitivity to oxidative stress, potentially due to decreased capacity to store iron, reduced ability to proliferate inside macrophages and decreased expression of virulence factors such as LLO (McLaughlin et al. 2011).

In addition to its role in virulence, the ferritin protein in *L. monocytogenes* was considered the principal cold shock protein, necessary for the resistance to both heat and cold shock, allowing the survival of *Listeria* in food matrices that are subject to temperature variations (Dussurget et al. 2005, McLaughlin et al. 2011).

7. *SALMONELLA ENTERICA* SPP TYPHIMURIUM

Salmonella enterica spp Typhimurium (*S.* Typhimurium) is a member of *Enterobacteriaceae* family and *Salmonella* genus, *Salmonella enterica* species and *Salmonella* enterica subspecies. This pathogen consists of a gram-negative, rod shaped, aerobic, flagellated and facultative intracellular bacterium (Wick 2011, Broz et al. 2012).

Salmonella are taken up via contaminated food and can infect a broad range of hosts. A distinctive feature of these bacteria is that it has adapted to survive in a strong inflammatory environment and uses such adaptation as a strategy to gain a growth advantage over the intestinal microbiota (Wick 2011, Broz et al. 2012).

Several routes may contribute to *Salmonella* penetration across the layer of intestinal epithelial cells. Bacterial crossing via M cells overlying Peyer´s patches is a predominant pathway, particularly for invasive bacteria. The invasion of intestinal epithelial cells causes gastrointestinal infections and is mediated by the type III secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (SPI1)(Ellermeier and Slauch 2008, Ibarra and Steele-Mortimer 2009). However, *S.* Typhimurium can also translocate across the colonic wall and promote systemic diseases (Wick 2011, Kortman et al. 2012).

7.1 IMMUNE RESPONSE AGAINST *S.* TYPHIMURIUM

The first line of defense against *Salmonella* infection consists of physical barriers, cellular barriers, such as intestinal epithelial cells, and chemical barriers, including a thick layer of mucous that covers the surface of intestinal epithelium and that is composed by mucins, a family of glycoproteins secreted by goblet cells (Wick 2011, Broz et al. 2012). Furthermore, intestinal epithelial cells, such as paneth cells, release antimicrobial peptides, including α and β-defensins, lysozyme and phospholipase A2 (sPLA2) as part of the inflammatory response to invading pathogens. These cells can also be induced to secrete other types of antimicrobial peptides, such as c-type lectins RegIIIβ/γ or angiogenin 4 (ANG4) in response to microbial PAMPs. Antimicrobial peptides disrupt the integrity of the bacteria cell membranes (Broz et al. 2012). RegIII γ secretion can also contributes to the clearance of other intestinal pathogens such as *L. monocytogenes* (Broz et al. 2012).

After crossing epithelial, cellular and chemical barriers, *S*. Typhimurium finds another obstacle: the resident immune cells, including macrophages and dendritic cells that remove invading microorganisms by phagocytosis and alert other immune cells of the infection, either directly or by secreting pro-inflammatory cytokines. Increased numbers of DCs and its

maturation are essential early events in the response to *Salmonella* infection and are key steps to start the adaptive immune response against this pathogen (Wick 2011, Broz et al. 2012).

Following phagocytosis, *S*. Typhimurium express their virulence-associated SPI-2 T3SS (type III secretion system encoded by *Salmonella* pathogenicity island-2) complex to establish themselves in an intracellular compartment designated as *Salmonella* containing vacuole (SCV)(Ibarra and Steele-Mortimer 2009, Teixido et al. 2011, Broz et al. 2012). Although S. Typhimurium remains partially hidden within its intracellular niche, it cannot completely escape from host cell sensing because all monocytic cells express PRRs that detect bacteria PAMPs. PAMPs of extracellular *Salmonella* activate several TLRs, including TLR 1, 2 and 6 that are activated by lipoproteins, TLR4 induced by LPS, TLR5 turned on by *Salmonella* flagellin FliC and finally TLR9 activated by CpG-rich repetitive elements in *Salmonella* DNA. The recognition of PAMPs by TLRs initiates signalling cascades, leading to the expression of pro-inflammatory cytokines such as IL-22, IL-23, IL-1β or TNF-α (Figure 6A)(Wick 2011, Broz et al. 2012).

Macrophages and lymphocytes are also able to recognize the presence of intracellular *Salmonella* PAMPs in the cytosol by NLRs which induces IL-23 expression, as well as the activation of NLRC4 also known as IPAF, a cytoplasmic flagellin sensor that initiates inflammasomes assembly, an important element in the innate immune response to *Salmonella*. The assembly of inflammasomes activates caspase-1 that, in turn, promotes the secretion of mature IL-1β and IL-18 (Figure 6A) (Mariathasan et al. 2004, Akira et al. 2006). The activation of caspase-1 mediated by SPI-1 and by NLRP3 another cytosolic receptor of the NLR family may also contribute to IL-18 secretion (Figure 6A) (Wick 2011, Broz et al. 2012, Claes et al. 2014).

Detection of bacterial PAMPs triggers an acute, mucosal inflammation in the gut during infection with *S.* Typhimurium. This condition is induced in response to the entry of *Salmonella* in the intestinal epithelium and survival in tissue macrophages (Broz et al. 2012).

Inflammatory response is amplified by IL-18 and IL-23 through paracrine signaling mechanisms (Broz et al. 2012). IL-18 induces the release of IFN-γ from mucosa resident T cells which, in turn, activates microbicidal mechanisms in infected macrophages, promoting the internalization of bacteria and stimulates its elimination by various mechanisms, including RNI, generated by iNOS (Mastroeni 2002, Charles et al. 2008, Nairz et al. 2008). Bacterial replication is restricted by RNI antimicrobial peptides that have the potential to damage bacterial DNA (Nagy et al. 2014).

Moreover , IL-23 induces the release of IL-22 and IL-17 from mucosa resident T cells (Broz et al. 2012). These cytokines increase the production of mucins and antimicrobial peptides and promote the release of CXC chemokines by intestinal epithelial cells that, in turn,

attract neutrophils into the mucosa, which is crucial to prevent the dissemination of *S.* Typhimurium from the gut (Figure 6B and 6C) (Broz et al. 2012).

Although neutrophils are important cells in host defense against *Salmonella*, their influx can also lead to intestinal tissue damage, sometimes associated with necrosis, resulting in loss of epithelial cell barrier function and, consequently, in an increase of inflammation (Broz et al. 2012).

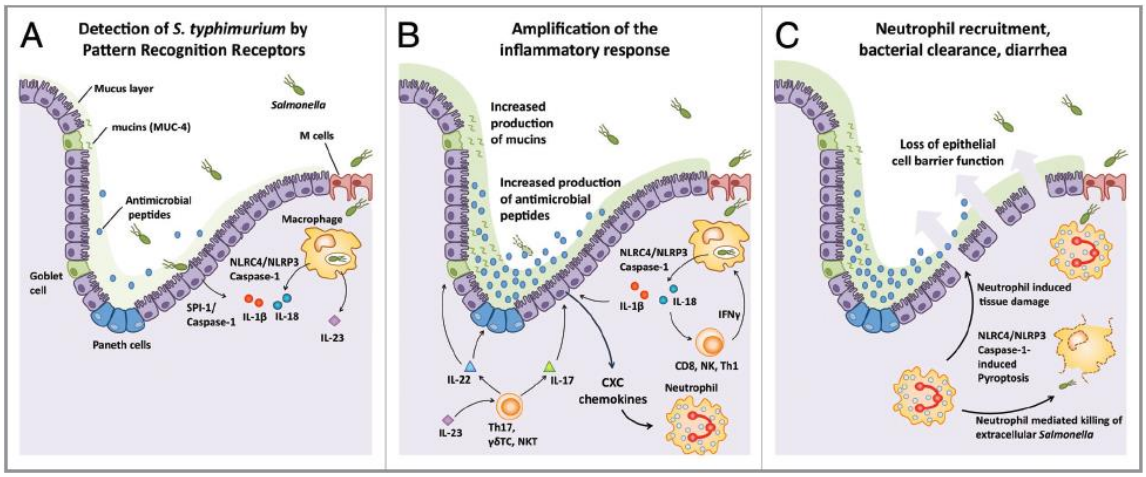

Figure 6: The host immune response against *Salmonella*. (A) *Salmonella* that invade mucosa are detected by PRRs (TLRs and NODs). This recognition induces a transcriptional response and leads to the expression of pro-inflammatory cytokines such as IL-23. Intracellular bacteria are also able to induce the assembly of NLRC4/NLRP3 inflammasomes that activate caspase-1 and promotes the secretion of mature IL-1β and IL-18. (B) Amplification of inflammatory response is mediated by IL-18 and IL-23 through paracrine signaling. C) Neutrophils recruitment is crucial to kill *Salmonella*, however neutrophil influx can also promote tissue damage, leading to the loss of epithelial cell barrier function - Adapted from (Broz et al. 2012).

7.2 *S.* TYPHIMURIUM STRATEGIES TO AVOID HOST IMMUNE DEFENSE

Salmonella Typhimurium, as intracellular pathogen has a machinery to promote cell invasion. This bacterium has in its genome an invasin operon (inv A - H) that encodes for factors that regulate their entry into host cells (Hornef et al. 2002).

In order to overcome the chemical barrier generated by antimicrobial peptides, *S.* Typhimurium developed several methods to sense the host responses, upregulating the corresponding virulence factors in order to induce inflammation during intestinal colonization. This mechanism allows it to gain a growth advantage over the resident intestinal microbiota. *Salmonella* has a two component signal transduction system, the PhoP-PhoQ that senses the presence of cationic antimicrobial peptides, divalent cations as well as the low pH. Increased resistance against antimicrobial peptides is achieved by modifications of lipid A either by acylation (PagP) or addition of an aminoarabinose moiety (Pmr system) (Broz et al. 2012).

Furthermore, TLR signaling can also be used by *S*. Typhimurium to enhance its virulence. For example, TLR4 signaling induced by *Salmonella* LPS impairs expression of homeostatic chemokines involved in the organization of lymph node architecture, which reduces the efficiency of host adaptive immune response and compromises bacterial clearance (Wick 2011).

Additionatly, when *S*. Typhimurium is established within the SCV, it can replicate before exiting the cell and infecting new host cells. Furthermore, this intracellular compartment able to this bacterium hides from many extracellular detection mechanisms and avoids its fusion with acidic lysosomes (Pan et al. 2010, Broz et al. 2012). TLR signaling is required for acidification of SCV which is necessary for *Salmonella* express virulence genes that, in turn, are critical for intracellular replication of *Salmonella* and hence for its survival. Thus, the TLR signaling, which is usually considered a host defense mechanism, can also contributes to *S*. Typhimurium virulence (Wick 2011).

On the other hand, MsbB *Salmonella* enzyme modifies LPS, a bacterial PAMP. MsbB attaches a myristic acid residue onto lipid A resulting in a hexa-acylated LPS. Moreover, the acyl transferase PagP adds a palmitic acid onto the complete lipid A making hepta-acylated lipid that is less recognized by TLR4 (Claes et al. 2014).

Salmonella can also inhibit the the fusion of the phagocytic lysosomes with the phagosome thus, preventing the discharge of lysosomal contents into the phagosome environment (Hornef et al. 2002, Coombes et al. 2004).

Finally, the pH developed in the phagosome after engulfment of *S*. Typhimurium induces bacterial gene products that are essential for their survival in macrophages (Coombes et al. 2004).

7.3 IRON AND *S*. TYPHIMURIUM

S. Typhimurium modulates the gene expression of virulence factors, adapting it to each stage of the infection process and depending on the free iron concentration found in the host intestinal epithelium. The pathogen regulates these genes through the Fur protein, which acts as a sensor of iron levels in its surroundings (Teixido et al. 2011).

It is known that iron overload states increase the risk of infection with *Salmonella* and allelic variations in the macrophage metal transporter Nramp1 (Slc11a1) affect the resistance to these bacteria, probably by influencing intraphagosomal iron concentrations (Chlosta et al. 2006).

Additionatly, in 2006, Kortman and co-workers showed that the adhesion of *S*. Typhimurium to intestinal epithelial cells and the capacity to translocate across the epithelial

monolayer and cause a systemic infection significantly increased with high iron concentrations (Kortman et al. 2012).

7.3.1 LIMITATION OF IRON AVAILABILITY FOR *S.* TYPHIMURIUM

In 2006, Chlosta S. and collaborators showed that high levels of iron exporter ferroportin inhibited the growth of *Salmonella*. As ferroportin is located on the plasma membrane and intracellular vesicles in primary macrophages, it can inhibit bacterial growth by either iron deprivation or iron toxicity, depending on whether its iron export activity function predominantly at the plasma membrane or the membrane of *Salmonella*-containing vacuole (Chlosta et al. 2006, Achard et al. 2013).

Furthermore, in infections with intra-macrophage pathogens, including *S.* Typhimurium, IFN-γ, a T_{H1} cytokine, has a pivotal importance in host defense mechanisms against bacteria. Firstly, IFN-γ causes a significant reduction of endocytic uptake of iron-transferrin complex by TFR-1, since it significantly reduces the expression of this receptor. Secondly, IFN-γ increases iron efflux by increased expression of ferroportin, the iron exporter. This way, IFN-γ reduces the iron content within phagocytes infected with *S*. Typhimurium and, subsequently, it restricts iron acquisition by these intracellular bacteria while concomitantly promote the NO and TNF-α production (Ludwiczek et al. 2003, Schaible and Kaufmann 2004, Nairz et al. 2007, Nairz et al. 2008, Achard et al. 2013).

Finally, mammals have also evolved mechanisms, that specifically interfere with siderophore-mediated iron uptake by microbes, such as the lipocalin 2 (Lcn2) protein, which is secreted by neutrophils and macrophages in response to bacterial infections and captures iron from microbial Ent siderophore. Iron may thus be delivered to mammalian cells via lipocalin 2 receptor (LcnR)(Flo et al. 2004, Nairz et al. 2008, Muller et al. 2009, Holden et al. 2014).

7.3.2 MECHANISMS DEVELOPED BY *SALMONELLA* TO ACCESS MAMMALIAN IRON RESOURCES

S. Typhimurium is a facultative intracellular microorganism able to invade macrophages and use these cells for multiplication and systemic spreading. However, within phagocytes, *S.* Typhimurium has limited access to extracellular mammalian iron resources, forcing this bacterium to develop mechanisms for obtain sufficient amounts of iron from intracellular host iron sources (Nairz et al. 2008, Pan et al. 2010).

To acquire sufficient iron from the host, *S*. Typhimurium produces small secondary metabolites called siderophores that compete with host proteins for iron. *S*. Typhimurium secretes two catecholate siderophores: the enterochelin (enterobactin - Ent) and salmochelin,

a C-glucosylated form of enterochelin, allowing the bacterium to chelate ferric iron with high affinity and mobilize it for use (Hantke et al. 2003, Crouch et al. 2008, Muller et al. 2009, Holden et al. 2014). The ability to produce siderophores enhances the survival of *Salmonella* in macrophages mainly at the early stages of infection, since salmochelins and enterobactins protect *S*. Typhimurium against ROS, having an antioxidant effect (Achard et al. 2013).

S. Typhimurium secretes enterobactin and salmochelin from cytoplasm to the extracellular space by IroC. Outside of the cell, siderophores compete with host proteins for the acquisition of ferric iron (Braun 2001, Hantke et al. 2003, Lin et al. 2005, Nairz et al. 2007, Nairz et al. 2008, Muller et al. 2009). Enterobactin can also be exported by the major facilitator superfamily pump EntS (Crouch et al. 2008). Ferric iron complexed with salmochelin and Ent is then taken up by catecholate siderophore receptors present in cell outer membrane, including IroN and FepA, into the periplasm, where the IroE protein degrades cyclic ferric salmochelin to the linear form which, in turn, is transferred to the cytoplasm by FepBCDG ABC transporter, present in cytoplasmic membrane (Figure 7) (Hantke et al. 2003, Crouch et al. 2008, Muller et al. 2009). In periplasm, Fe-Ent also requires the FepBCDG ABC transporter to be delivered to the cytoplasm (Raymond et al. 2003, Payne 2004, Crouch et al. 2008).

Inside the cell, the Fe-Ent and Fe-salmochelin complexes may be cleaved by Fes esterase and IroD and the degradation products glucosylated 2,3-dihydroxybenzoylserine (GDBS) are exported and may be reused as siderophores. In cytoplasm, IroB is the enzyme responsible for glucosylate enterobactins and produce salmochelins (Hantke et al. 2003, Crouch et al. 2008, Muller et al. 2009, Achard et al. 2013).

Indeed, the majority of siderophores produced by *Salmonella* Typhimurium are salmochelins (Crouch et al. 2008).

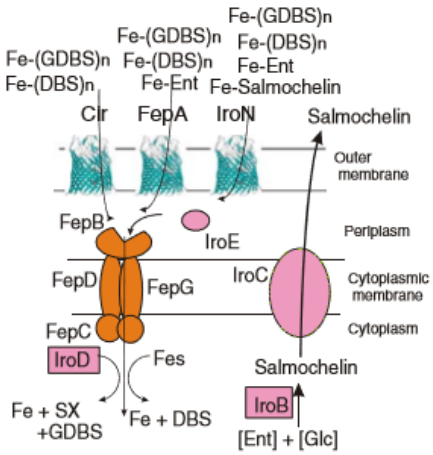

Figure 7: Iron acquisition system by *S*. Typhimurium. Fe-Ent and Fe-salmochelin complexes are transported across the outer membrane mainly by FepA and IroN receptors. The Fe-siderophores complexes are then transported across cytoplasmic membrane by FepBCDG transporters. In cytoplasm

siderophores containing iron are degraded by IroD and Fes esterases, Ent is glucosylated by IroB and secreted with the help of IroC – Adapted from (Hantke et al. 2003, Muller et al. 2009)

Glucosylation of enterobactin significantly alters its chemical properties (Luo et al. 2006). The reduced hydrophobicity can help to avoid the sequestration by lipid-rich membranes, thereby enhancing its iron-chelator function. On the other hand, the glucosylation of enterobactin leads to a decreased binding with the host Lcn2, an antimicrobial peptide secreted by macrophages that binds Ent to disrupt bacterial iron acquisition, to inhibit bacterial replication and to promote acute inflammation during colonization. Thus, salmochelin allows iron delivery to bacteria despite the presence of Lcn2 (Luo et al. 2006, Nairz et al. 2007, Muller et al. 2009, Broz et al. 2012, Frawley and Fang 2014, Holden et al. 2014).

On the other hand, *Salmonella* RstA protein activates the expression of feoAB operon which encodes the FeoB, a transmembrane transporter of Fe^{2+}, which allows more iron to be imported into the bacterial cell (Braun 2001, Jeon et al. 2008, Nairz et al. 2008, Nagy et al. 2014).

Invasion of intestinal epithelium is facilitated by T3SS, encoded in SPI1. SitABCD operon is also encoded in SPI1, being induced after invasion of intestinal epithelium and under iron-deficient conditions. SitABCD is an ABC transporter that allows the acquisition of ferrous iron (Janakiraman and Slauch 2000, Braun 2001, Achard et al. 2013, Frawley and Fang 2014).

Intriguingly, *S.* Typhimurium is dependent on the acquisition of both ferric and ferrous iron for full virulence (Nairz et al. 2007).

In *S*. Typhimurium, the transcription of genes that encode enzymes for the synthesis of siderophores and iron transport proteins is regulated by Fur protein (Braun 2001, Ellermeier and Slauch 2008, Jeon et al. 2008, Achard et al. 2013).

II. HYPOTHESES/ AIMS

The present work is focused on the study of the iron metabolism in the context of host-pathogen interaction. Hepcidin has been considered as the key regulator of host iron metabolism during infection. However, previous work from our group has shown that in certain types of bacterial infections, such as infection with *Mycobacterium avium,* the alterations on iron metabolism were not dependent on hepcidin expression (Rodrigues et al. 2011).

Thus, our main questions are: Do different types of bacteria induce different alterations in the host iron metabolism? Are those differences based on different hepcidin levels?

In order to answer these questions, the work was divided into two tasks:

1. Iron metabolism regulation in the host upon *Listeria monocytogenes* infection. As previous data from the group showed that *Mycobacterium avium* infection and consequent anaemia in the host is not dependent on hepcidin levels (Rodrigues et al. 2011), in this task, we decided to test another gram-positive and intracellular bacterium: *Listeria monocytogenes*. Some of the work presented here was already ongoing in the laboratory. During the course of my master's thesis it was decided to narrow some time points and that is the main reason why in this thesis, data from two different experiments are presented herein.

2. Iron metabolism regulation in the host upon *Salmonella* Typhimurium infection. In the second task, we decided to study a gram negative bacterial infection in order to know whether alterations in iron metabolism are dependent on the type of bacterial infection and whether hepcidin is involved in these alterations.

III. MATERIALS AND METHODS

1. INSTITUTION

Experimental procedures were performed at the Institute for Molecular and Cell Biology (IBMC) • Institute of Biomedical Engineering (INEB) associate laboratory (Porto, Portugal), at the Unit of Infection and Immunity, in the Laboratory of Iron and Innate Immunity.

2. CHEMICALS

All chemicals used in this work are of the highest analytical grade and were purchased from Sigma Aldrich Co (St. Louis, MO, USA), unless otherwise specified. Aqueous solutions were prepared in distilled water.

3. ANIMALS

Male C57BL6 mice were bred at the IBMC and used at 12 to 16 weeks-old. All mice were kept at the IBMC animal facility and housed in type III cages (Tecniplast, Buguggiate VA, Italy) with irradiated corn cob bedding (Ultragene, Porto, Portugal) and environmental enrichment with ad libitum access to sterile mucedola diet (Ultragene, Porto, Portugal) and autoclaved bi-distillated water. The animals were maintained at constant temperature (22^{o}C) and humidity with a 12h light/dark cycle. Animal maintenance, handling and sacrifice were conducted according to the rules of the IBMC animal ethics committee and followed the procedures approved by the Federation of European Laboratory Animal Science Associations (FELASA). The author of this master thesis completed the FELASA (category B) course. The project supervisor and colleagues involved in the experiments are credited by FELASA (category C) for animal experimentation.

4. BACTERIA

4.1. LISTERIA MONOCYTOGENES

Listeria monocytogenes EGDe was gently provided by Didier Cabanes (Molecular Microbiology group of IBMC). *Listeria* monocytogenes was pre-cultured in brain heart infusion (BHI) broth medium (BD biosciences, San Jose, CA, USA) overnight at 37^{o}C, under agitation (Figure 8). Afterwards the bacterial suspension was diluted (1:100) and incubated to mid-log phase at 37^{o}C until the optical density (O.D.) to be approximately 0.6-0.7. Next, bacteria were harvested by centrifugation and washed 3 times in ice-cold PBS at 5000 rpm for 5 minutes at 4^{o}C. Aliquots of the bacterial suspension were frozen at -80^{o}C prior to use. The quantification

of *L. monocytogenes* inoculum was performed in brain heart fusion (BHI) agar medium (BD biosciences, San Jose, CA, USA).

4.2. *SALMONELLA* TYPHIMURIUM

Salmonella Typhimurium strain ATCC 14028 was kindly provided by Luisa Peixe (Faculdade de Farmácia, Universidade do Porto). *Salmonella* Typhimurium was grown to mid-log phase in Tryptic Soy Broth (TSB) medium (Conda, Torrejón de Ardoz, Madrid, Spain) for 6h at 37°C and 150 rpm (Figure 9).

Bacterial growth was confirmed by the measurement of the O.D. that should be approximately 0.2 to 0.3. Afterwards, bacteria were harvested by centrifugation at 1500 g, 4°C for 10 minutes in PBS twice, suspended in a small volume of PBS and frozen in small aliquots at -80°C until use. The quantification of *S.* Typhimurium inoculum was calculated few days after freezing and confirmed prior of infection to ensure that the injected amount in mice was appropriate. These procedures were performed with different dilutions of bacterial inoculum in plates with Salmonella Shigella agar (SS agar) medium.

5. EXPERIMENTAL DESIGN

5.1. *LISTERIA MONOCYTOGENES*

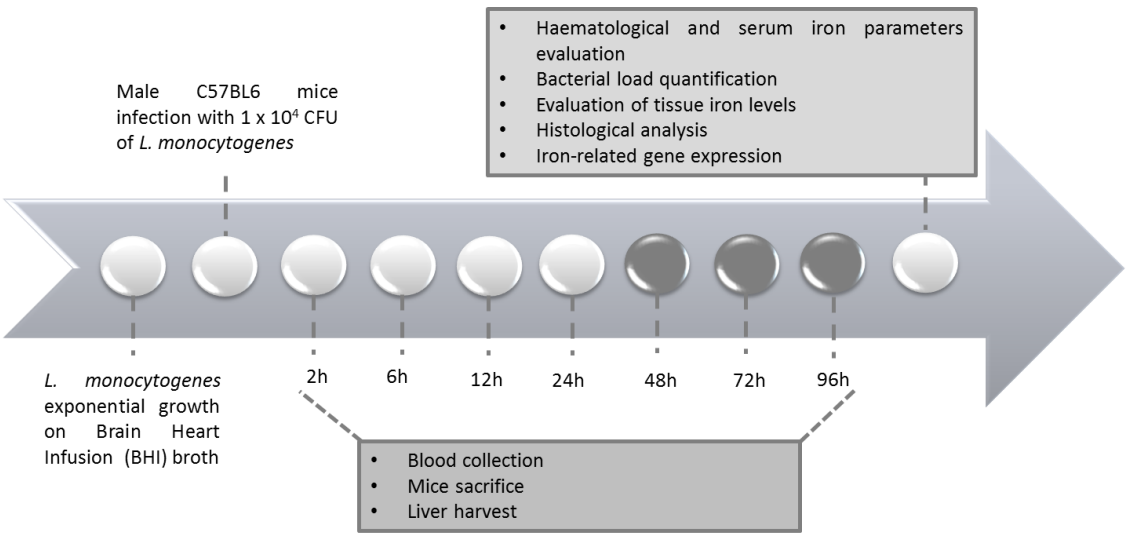

Figure 8: Experimental design of *Listeria monocytogenes* experiment. Animals were infected with 1 x 10^4 CFU of *L. monocytogenes*/animal or injected with an equivalent volume of saline solution. At each time-point, animals were sacrificed, blood and liver were collected in order to evaluate haematological parameters and iron metabolism alterations in host. Two distinct experiments were performed as shown in the figure: one experiment for earlier time-points (2 - 24h; white circles); a second experiment for 48h, 72h, and 96h (grey circles).

5.2. *SALMONELLA* TYPHIMURIUM

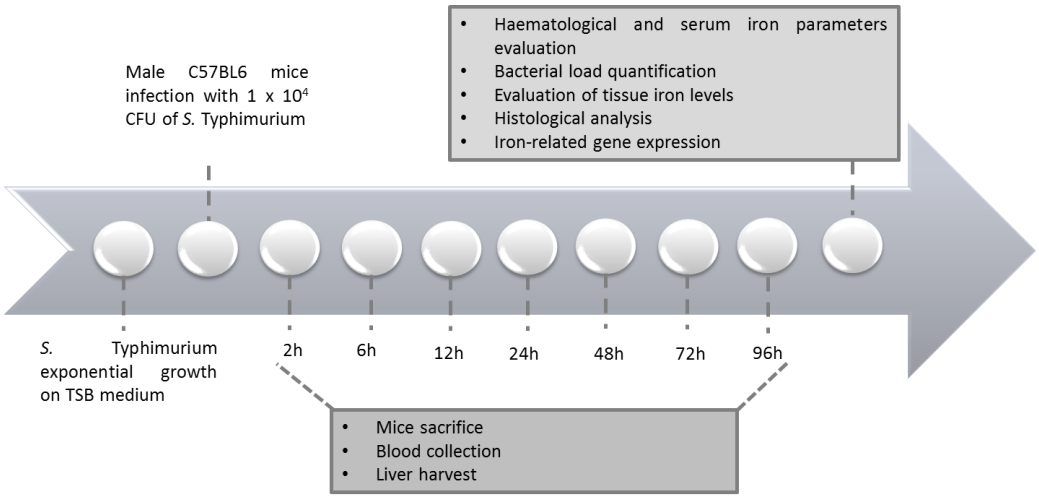

Figure 9: Experimental design of *Salmonella* Typhimurium experiment. Animals were infected with 1 x 10^4 CFU of *S*. Typhimurium/animal or injected with an equivalent volume of saline solution. At each time-point, animals were sacrificed, blood and liver were harvested in order to evaluate haematological parameters and iron metabolism alterations in host.

6. MOUSE INFECTION

Male C57BL6 mice were intravenously infected in a tail lateral vein with 1 x 10^4 CFU of either *L. monocytogenes* EGD or *S.* Typhimurium ATCC 14028 (Figure 8 and 9). Control mice received an equivalent volume of sterile saline solution (PBS) by the same route to neutralize the infection effects.

7. MOUSE SACRIFICE

Mice were anesthetized with isoflurane (B. Braun Medical, Portugal) and sacrificed with cervical dislocation at the following time points: 2h, 6h, 12h, 24h, 48h, 72h and 96 h after infection (Figure 8 and 9). At each time point, 4 to 7 animals were sacrificed and their livers were harvested for different analysis, including bacterial load quantification, gene expression evaluation, non-heme iron determination, histological alterations and examination of iron distribution in tissue. Liver samples collected for gene expression and iron analysis were quickly frozen in liquid nitrogen and stored at -80^{o}C until to use.

8. BACTERIAL LOAD QUANTIFICATION

Tissues were aseptically collected, weighted and homogenized in sterile PBS. Serial dilutions were performed and plated in duplicate into BHI agar medium or SS agar medium for

L. monocytogenes and *S*. Typhimurium, respectively. The plates were incubated at 37°C for 24 hours, after which the colonies were counted. The number of CFU/liver was calculated taking into consideration the dilution at which colonies were counted, the total organ weight and weight of the organ fraction used for CFUs.

9. HAEMATOLOGICAL AND SERUM IRON PARAMETERS

Blood samples were collected by retro orbital puncture under anaesthesia and 150 µl were transferred to Ethylenediamine tetraacetic acid (EDTA) tubes (BD vacutainer, New Jersey, United States), in order to evaluate the erythron. Serum was obtained by 13 000 rpm centrifugation of the remaining blood. Erythron and serum iron parameters were determined in a certified laboratory (CoreLab, Centro Hospitalar do Porto, Portugal).

10. GENE EXPRESSION

10.1. RNA EXTRACTION FROM ANIMAL TISSUE

RNA extraction from mouse liver was performed with the PureLink RNA mini kit (Life technologies, Carsbad, CA, USA), following the manufacturer's instructions. Briefly, fresh lysis buffer with 1% 2-mercaptoethanol (Sigma Aldrich Co, St. Louis, MO, USA) was added to small pieces of tissue, which were homogenized with a VDI 12 rotor-stator homogeneizer (VWR International, Radnor, PA, USA) at room temperature. Homogenates were centrifuged at 4000 g for 5 minutes, the supernatant was transferred into a clean RNase-free tube and one volume of 70% ethanol was added to each sample.

Each sample was transferred to the spin cartridge with the collection tube and centrifuged at maximum speed for 1 minute at room temperature. The flow-through was discarded and, then, wash buffer was added to each spin cartridge. Tubes were centrifuged over again under the same conditions and the flow-through was discarded.

The next step consisted of adding DNase to each spin cartridge. The DNase was obtained from PureLink DNase Kit (Invitrogen™, Waltham, MA, USA) following the manufacturer's instructions.

Wash buffer was added, the tubes were centrifuged again under the conditions described above and flow-through was discarded. Then, wash buffer with ethanol was added to the spin cartridge, the tubes were centrifuged twice at maximum speed for 1 minute and the flow-through was discarded.

The final step consisted in adding 30 µl of Rnase-free water to the spin cartridge. Tubes were centrifuged at maximum velocity for 2 minutes to elute the RNA from the membrane-columns into recovery tubes which were stored at – 80°C prior to use.

10.2. CONVERSION OF RNA TO CDNA

The quantity of RNA was evaluated by UV absorbance at 260 nm in the nanodrop ND-1000 spectrophotometer (Thermo scientific, Wilmington, DE, USA).

The cDNA synthesis was performed with the NZY First-strand cDNA synthesis Kit (Nzytech, Lisbon, Portugal), following the manufacturer's instructions. Briefly, RNA was added to a mix with oligo (dT)s primers, dNTPs, $MgCl_2$, RT buffer, reverse transcriptase and a ribonuclease inhibitor. The reaction occurred with different temperatures and times of incubation as follows: 25°C for 10 minutes, 50°C for 30 minutes and the reaction was inactivated at 85°C for 5 minutes using the T100™ thermal cycle (Bio-Rad laboratories, Hercules, CA, USA).

Tubes were cooled on ice and NZR RNase H (E. coli) was added in order to degrade the RNA template in cDNA. Tubes were incubated at 37°C for 20 minutes and stored at -20°C until required.

10.3. REAL TIME POLYMERASE CHAIN REACTION (RT-PCR)

We investigated the expression of different genes: hepcidin antimicrobial peptide 1 (*Hamp1*), interleukin-6 (*Il6*), transferrin (*Tf*), H ferritin (*Fth1*), L ferritin (*Ftl*), and ferroportin (*Fpn1*). The Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) was used as a reference gene (housekeeping gene).

A specific pair of primers (forward primer and reverse primer) from STAB Vida (Lisbon, Portugal) was used. The primers sequences used in this work are presented in Table 2.

All reactions were performed in the presence of cDNA samples, iQ™ SYBR® Green Supermix (Bio-Rad laboratories, Hercules, CA, USA), forward primer and reverse primer and RNase-free water (Invitrogen™, Waltham, MA, USA).

In the RT-PCR, 96-well clear PCR plates from Bio-Rad laboratories (Hercules, CA, USA) were used. PCR analysis was performed using the IQ™5 Multicolor Real Time PCR detection system (Bio-Rad laboratories, Hercules, CA, USA). Firstly, the cDNA was heated up to 94°C. In the second step, the temperature was lowered to 59°C. In the last, the temperature was raised to 72°C. The cycle described above was repeated 40 times.

Baseline thresholds were calculated by the Bio-Rad iQ5 program and the threshold cycles (CT) were used in the fold change ($2^{-\Delta\Delta Ct}$) method, where ΔΔCt = ΔCt [test sample] – [ΔCt control].

Table 2: Primers used in RT-PCR.

GENE	PRIMER SEQUENCE
Hprt	For - 5' GGT GGA GAT GAT CTC TCA AC 3'
	Rev - 5' TCA TTA TAG TCA AGG GCA TAT CC 3'
Hepcidin (*Hamp1*)	For – 5' CCT ATC TCC ATC AAC AGA TG 3'
	Rev – 5' AAC AGA TAC CAC ACT GGG AA 3'
IL-6 (*Il6*)	For - 5'TGC AAG AGA CTT CCA TCC AG 3'
	Rev – 5' CAT TTC CAC GAT TTC CCA GAG 3'
Transferrin (*Tf*)	For - 5' ACC TGG AAC AAC CTG AAA GG 3'
	Rev - 5' GGC CAA TAC ACA GGT CAC AG 3'
Ferritin H (*Fth1*)	For - 5' GCT GAA TGC AAT GGA GTG TGC A 3'
	Rev - 5' GGC ACC CAT CTT GCG TAA GTT G 3'
Ferritin L (*Ftl*)	For - 5' ACC TAC CTC TCT CTG GGC TT 3'
	Rev - 5' TGG CTT CTG CAC ATC CTG GA 3'
Ferroportin (*Fpn1*)	For - 5' TTG GTG ACT GGG TGG ATA AGA ATG C 3'
	Rev - 5´CGC AGA GGA TGA CGG ACA CAT TC 3'

11. NON-HEME IRON DETERMINATION IN TISSUES

Non-heme iron determination in liver was performed by the batofenantroline method. Small liver pieces were weighted, placed in white iron-free teflon cups (ACV – advanced composite vessel, CEM corporation, Matthews, NC, USA) and dried in a CEM-MDS 2000 microwave digestor oven (CEM corporation, Matthews, NC, USA) for 2 hours. Dried liver samples were accurately weighed, transferred into a new tube and digested with an acid mixture (30% of 36,5% hydrochloric acid and 10% of trichloroacetic acid) for 20 hours at 65^{o}C

in an incubator (Binder, Tuttlingen, Germany). A blank was also prepared in the same way but omitting the tissue.

After incubation, the samples were left to cool down to room temperature and 50-250 µl of supernatant were transferred to a new eppendorf tube which already contained the working chromogen reagent (WCR): 5 volumes of distillated H_2O, 5 volumes of saturated sodium acetate and 1 volume of the 0,1% chromogen reagent (see appendix for detailed composition). The final volume was completed with deionized water. The blank and the standard were also prepared. The blank contained the WCR, the acid mixture incubated with the samples (blank) and deionized water. The standard was prepared by adding WCR, deonised water and working iron standard solution (WISS): 36,5% Hydrochloric acid, stock iron standard solution and deonised water (see appendix for detailed composition).

The colour of the product produced by the reaction between the WCR and the iron was measured by spectrophotometry at 535 nm against distilled water blank in a PowerWave XS microplate spectrophotometer (Biotek, Winooski, VT, USA).

Iron levels in dry tissue (µg/g dry tissue) were calculated by the following equation:

$$Liver\ iron\ \frac{\mu g}{g} dry\ tissue = \frac{A_T - A_B}{A_{S-A_B}}\ x\ \frac{Fe_S}{W_S}\ x\ \frac{\frac{V_f}{V_T}}{\frac{V_f}{V_S}}$$

Legend: A_T (Test sample absorbance), A_B (Blank absorbance), A_S (Standard absorvance); Fe_s (Standard iron concentration = 11.169 µg/Fe ml), W_s (sample dry weight (g)); V_f (final volume of acid mixture after overnight incubation (1300 µl)), V_T (Test sample volume = 50 – 100 µl), V_S (standard volume = 150 µl of WISS).

12. HISTOLOGICAL ANALYSIS

Liver sections were fixed in 10% neutral buffered formalin (Bio-optica, Milan, Italy) overnight at room temperature. Subsequently, the samples were washed, dehydrated through a crescent ethanol series, cleared with clearRite and infiltrated with paraffin in STP 120 Spin Tissue Processor (Thermo scientific, Waltham, MA USA). Tissue sections were then embedded in paraffin blocks through Microm EC 350 Modular tissue embedding center (Thermo scientific, Waltham, MA USA). Three µm thickness tissue sections were obtained in Microm HM335E microtome (Microm International GmbH, Walldorf, Germany).

12.1. PERLS PRUSSIAN BLUE STAINING

The ferric iron distribution in liver was detected by Perls Prussian blue staining. Paraffin sections were deparaffinized in xylol (Fisher scientific, Loughborough, UK) and

hydrated with decreasing concentrations of ethanol namely 100% (Merck Millipore, Darmstadt, Germany), 96% (Aga, Prior Velho, Portugal), 70% and 50%. Sections were rinsed with distilled water and incubated in Perls Prussian blue staining solution: 2% Potassium hexacyanoferrat (II) trihydrate, 2% Hydrochloric acid (see appendix for the detailed composition) for 30 minutes, followed by washing with distilled water.

Counterstaining was obtained with neutral red dye (see appendix for the detailed composition). Tissue samples were dehydrated with increasing concentrations of ethanol, namely 70%, 96% and 100% and cleared with xylol. The slides were, then, mounted with Entellan (Merck Millipore, Darmstadt, Germany).

12.2. HEMATOXYLIN-EOSIN (H&E) STAINING

Deparaffinization and rehydration of tissue sections was performed as described in section 12.1. Tissue sections were stained with hematoxylin solution modified according to Gill III (Merck Millipore, Darmstadt, Germany) for nuclear staining and rinsed in 0,1% HCl solution: 37% HCl (Sigma Aldrich Co, St. Louis, MO, USA) and distillated water.

The next step was based on differentiation under running water. Counterstaining of proteins, collagen, keratin or connective tissue was performed by staining of tissue sections with aqueous Eosin Y solution 0.5 % (see appendix for detailed composition).

After, tissue sections were rinsed under water and dehydrated with an increasing proportion of ethanol, namely 50%, 70%, 96% and 100% and cleared with xylol. Finally, the slides were mounted with Entellan (Merck Millipore, Darmstadt, Germany).

Olympus optical microscope CX31 with DP 25 camera (Tokyo, Japan) was used to observe sections stained with hematoxylin-eosin staining. Pictures were obtained using the imaging software Cell^B version 5.1 for windows at magnification of 100 x and 400x.

13. STATISTICAL ANALYSIS

Data were analyzed using the GraphPad Prism software version 6.02 for Windows. Data are expressed as mean ± standard deviation (SD) for the number of experiment/animals indicated in the legend of the figures. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc test. T-test was used when only two conditions were compared. Significance was accepted when p value < 0.05 was obtained. Data are represented with * when $p < 0.05$, ** when $p < 0.01$ and *** when $p < 0.001$.

IV. RESULTS

1. Bacterial infections with *L. monocytogenes* and *S*. Typhimurium have different kinetics in the mouse

Each mouse was infected with $1x10^4$ CFU of pathogen (*L. monocytogenes* or *S*. Typhimurium) or with an equivalent volume of vehicle for control mice as described in the material and methods section. In order to evaluate bacterial load, mice were sacrificed at each time point, the liver was aseptically collected and a fraction was homogenized. Serial dilutions of homogenates were performed and plated in BHI agar medium or SS agar medium for *L. monocytogenes* and *S*. Typhimurium, respectively.

Bacterial colonies were allowed to grow at 37°C for 24 hours. The values of CFU/liver were obtained by the average number of colonies counted in each duplicate, having in consideration the dilution at which colonies were counted, the total tissue weight and CFUs fraction weight.

A slight decrease in liver bacterial load was observed during the first hours after infection in animals infected with both *L. monocytogenes* and *S*. Typhimurium (Figure 10). This decrease may be explained by an attempt of the host to battle against the infection, which was not completely successful. From 6 to 72 hours after infection, the liver bacterial load increased in both experiments. Animals infected with S. Typhimurium died within 96 hours post-infection (Figure 10B) whereas animals infected with *L. monocytogenes* experienced a slight decrease in bacterial load in the last 24 h of the experiment (Figure 10A).

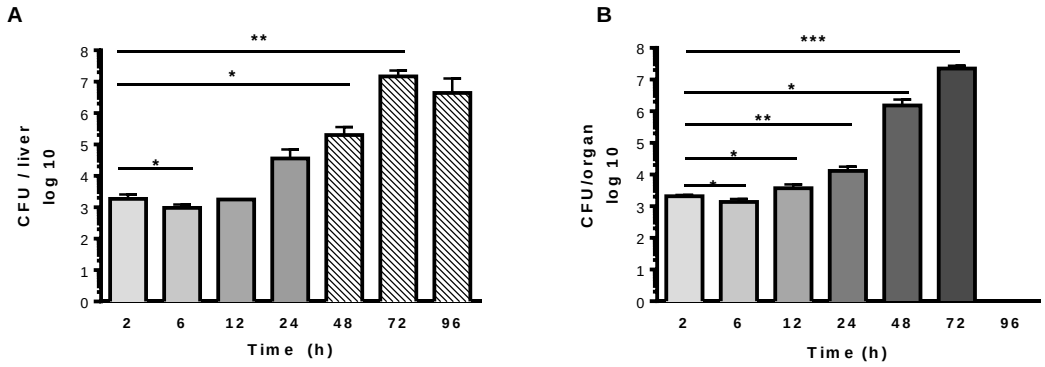

Figure 10: Liver bacterial load in animals infected with *L. monocytogenes* or *S.* Typhimurium. After sacrifice at different time points, liver was aseptically harvested in order to evaluate the bacterial load after *L. monocytogenes* (A) and *S*. Typhimurium infection (B). The graphs depict the mean ± SD of the log^{10} CFU/organ of 4 to 5 animals. * when $p<0.05$, ** when $p<0.01$ and *** when $p<0.001$ as shown by the horizontal lines in the respective graph. Pattern bars represent data from a non-simultaneous experiment**.**

2. Bacterial infection with *L. monocytogenes* and *S.* Typhimurium induces alterations in haematological parameters

At each infection time-point, blood samples were collected RBCs counts, haematocrit (HCT) and mean corpuscular volume (MCV) were assessed. The numbers of circulating red blood cells were significantly reduced in animals infected with *L. monocytogenes* for 2h, 12h, 48h and 96h after infection, when compared to non-infected control animals, with the highest reduction registered at 96h post infection (Figure 11A).

Furthermore, in mice infected with *L. monocytogenes*, the haematocrit showed significant reductions starting from 48h after infection until the end of experiment with a more marked reduction in the last 24 hours of infection (Figure 11B).

Finally, MCV did not show significant alterations during infection with *L. monocytogenes* (Figure 11C). The decrease of RBCs number and HCT indicate that these animals developed anaemia.

The animals infected with *S*. Typhimurium did not show statistically significant alterations in RBC numbers comparing to control mice, although at 72 hours post- infection a non-statistically significant decrease was observed (9.7±1.15 x 10^6 RBC/µL in control versus 8.9±0.3 x 10^6 RBC/µL in infected animals). Interestingly, at this infection time-point, mice infected with *S*. Typhimurium presented significant reductions in the haematocrit when compared to uninfected animals (Figure 11E).

On the other hand, MCV presented a statistically significant increase at 24h after infection with *S*. Typhimurium comparing with control animals (Figure 11F). Overall, these data suggest that mice infected with *S*. Typhimurium also have some manifestations of anaemia, although not as marked as that observed in mice infected with *L. monocytogenes*.

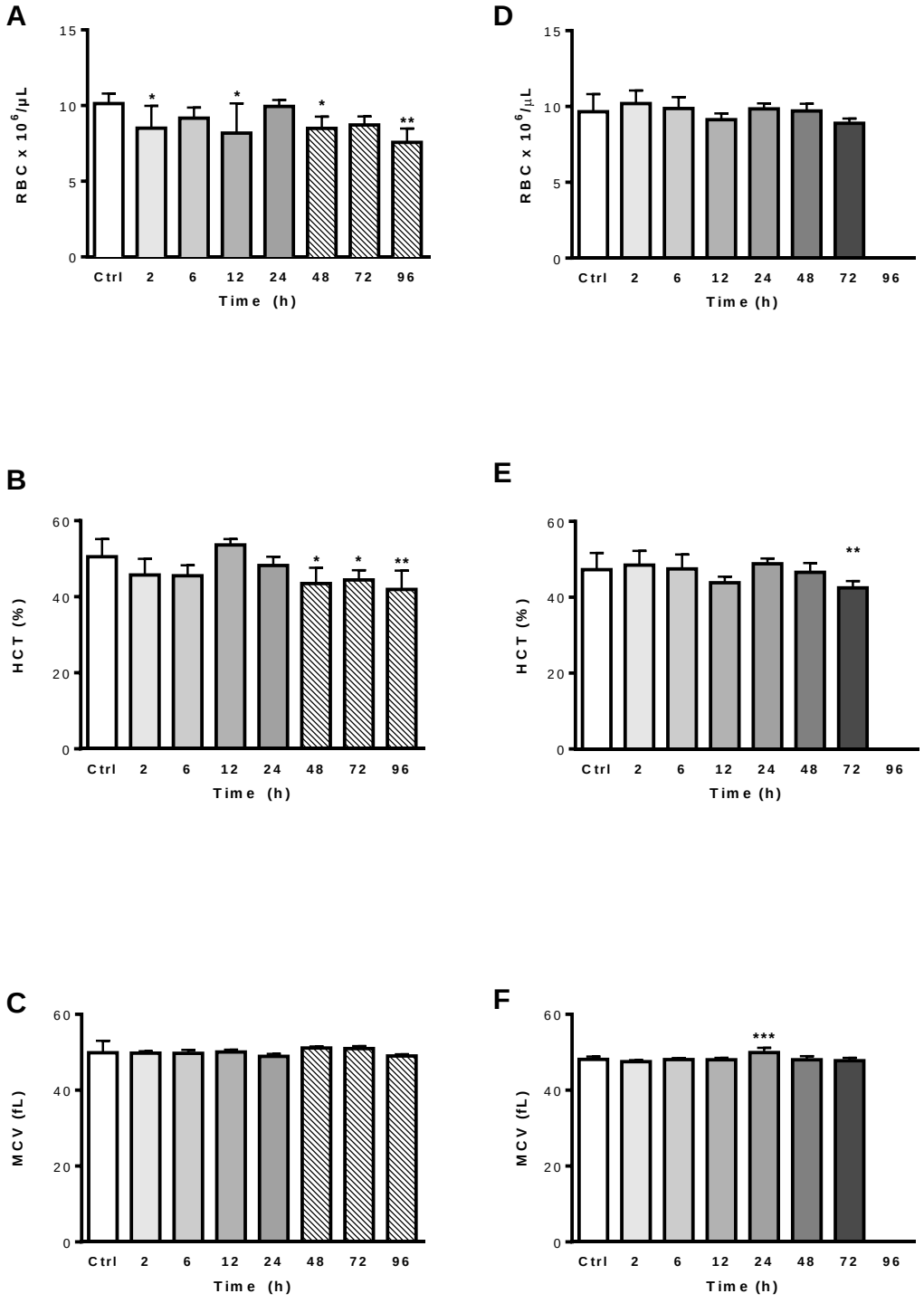

Figure 11: Infection with *L. monocytogenes* or *S.* Typhimurium induces alterations in haematological parameters. Haematological parameters were evaluated in mice infected with *L. monocytogenes* (A-C) or *S.* Typhimurium (D-F). The graphs show mean ± SD of 3 to 5 animals. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni post hoc test. *p<0.05, **p<0.01, ***p<0.001 when compared with control non-infected mice. Legend: RBC – red blood cells, HCT – haematocrit, MCV – mean corpuscular volume. Pattern bars represent data from a non-simultaneous experiment.

3. Infection with *L. monocytogenes* and *S*. Typhimurium induces alterations in host iron metabolism

Along with the analysis of haematological parameters, serum iron parameters were also investigated. Iron levels, percentage of saturated transferrin (sTRF), unsatured iron binding capacity (UIBC) and total iron binding capacity (TIBC) were determined in serum of animals infected with *L. monocytogenes* or *S*. Typhimurium and compared with control mice.

Serum iron levels represent iron released from enterocytes, macrophages and hepatocytes. In mice infected with *L. monocytogenes*, serum iron levels showed a statistically significant reduction at 24h and 48h after infection and a significant increase at 96h post infection when compared with reference uninfected animals (Figure 12A). On the other hand, in animals infected with *S.* Typhimurium, serum iron levels presented significant reductions at 6h, 12h and 48h after infection, being the most marked reduction observed at 6h post infection (Figure 12E). The decrease of serum iron levels during infection with *L. monocytogenes* and *S*. Typhimurium suggests that the host tries to decrease the levels of iron available for bacteria, in order to decrease its growth and virulence.

Transferrin saturation (TSAT) is a measurement of the iron content of circulating transferrin, the plasma protein responsible for iron transport. In accordance with reduction in serum iron levels, there was also a decrease in saturated transferrin in the serum for 24h, 48h and 72h after infection with *L. monocytogenes* (Figure 12B). During infection with *S*. Typhimurium, sTRF undergoes a significant decrease earlier than in infection with *L. monocytogenes*, namely from 6h after infection until the end of experiment, comparing with control animals (Figure 12F). The reduction of TSAT in *S*. Typhimurium infection is also in accordance with decreased iron levels in serum.

During *L. monocytogenes* infection, UIBC presented statistically significant increases starting from the 2nd day after infection, with the highest levels observed at 72h post infection (Figure 12C). On the other hand, in *S*. Typhimurium infection, the UIBC presented an increase during the course of the experimental infection, showing significant increases starting from 6h after infection when compared with uninfected mice (Figure 12G).

Finally, TIBC was significantly increased in the last 2 days of the experiment with *L. monocytogenes* (Figure 12D). Likewise, mice infected with *S*. Typhimurium showed significant increases at 48h and 72h after infection for TIBC, with the most significant increase at 72h post infection, comparing with control animals (Figure 12H).

The parameters presented so far, clearly show that there is a deregulation of iron homeostasis in the host upon infection with *L. monocytogenes* and *S*. Typhimurium, which was then detailed with more experiments.

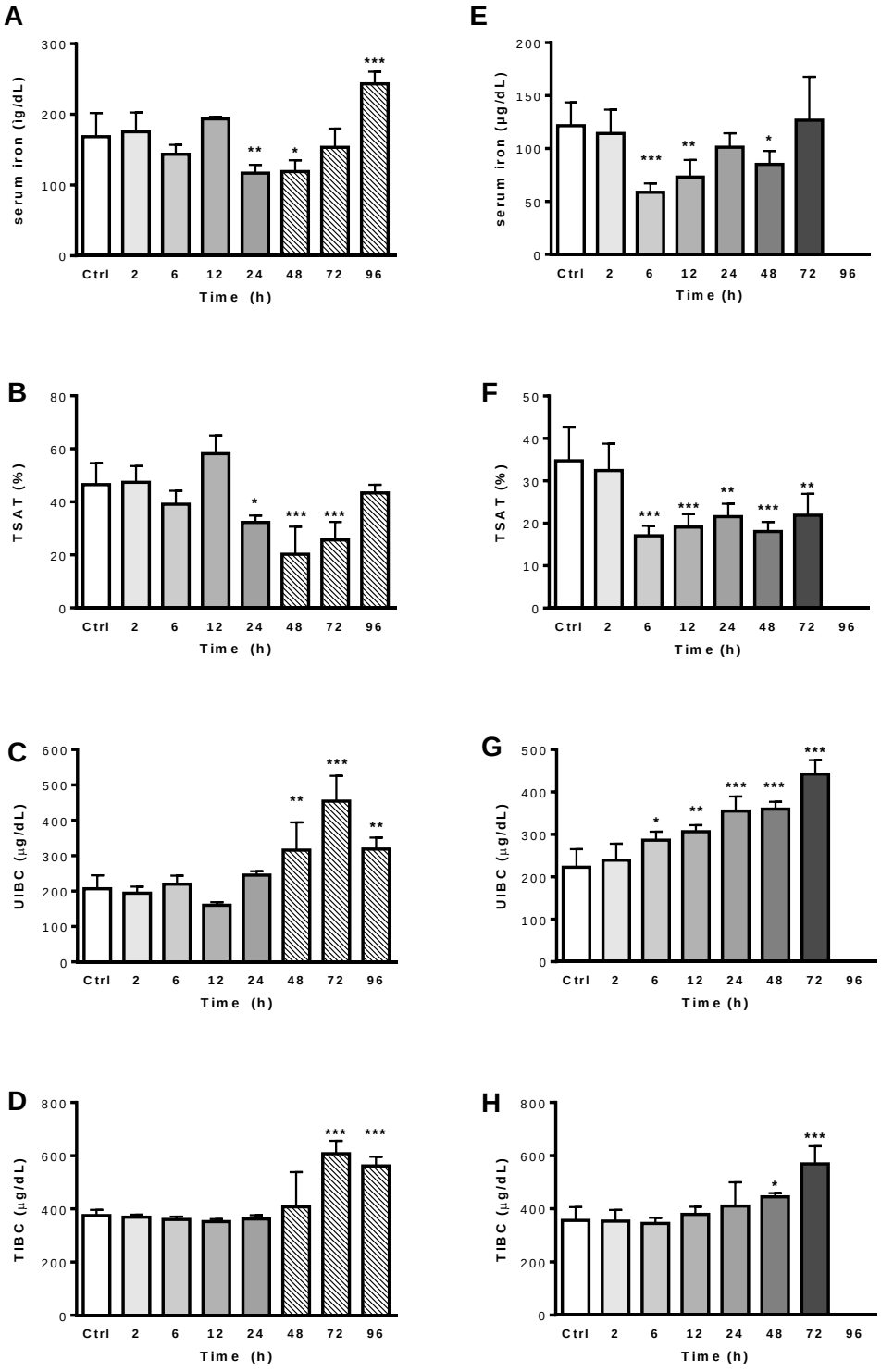

Figure 12: Serum iron parameters are altered in mice during infection with *L. monocytogenes* and *S*. Typhimurium. Infection with *L. monocytogenes* (A-D) and *S*. Typhimurium (E-H) induces alterations in iron metabolism of the host, observed by alterations in serum iron parameters. The graphs depict mean

± SD of 3 to 5 animals. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni post hoc test. *p <0.05, **p <0.01, ***p <0.001 when compared with control animals. Legend: TSAT – transferrin saturation, UIBC – unsatured iron binding capacity, TIBC – total iron binding capacity. Pattern bars represent data from a non-simultaneously experiment.

4. Infection with *L. monocytogenes* and *S*. Typhimurium modifies the expression of genes involved in iron metabolism

To investigate the mechanisms responsible for altered iron status during infection with *L. monocytogenes* and *S*. Typhimurium, the expression of genes involved in iron metabolism was analysed in liver lysates. Table 3 and Table 4 present the alterations in gene expression observed during infection with *L. monocytogenes* and *S*. Typhimurium, respectively.

Hepcidin (*Hamp1*), a peptide secreted by hepatocytes and macrophages in response to various inflammatory stimuli and iron overload presented a significantly increased (2-3 fold) expression at 6h, 48h and 72h after infection with *L. monocytogenes* (Table 3). In mice infected with *S*. Typhimurium, *Hamp1* presented an increased expression in the liver earlier in the infection (2h post infection) and higher (4-14 fold), when compared with *L. monocytogenes* infection. Significantly increased expression of *Hamp1* persisted for all time points tested in comparison with control animals with the exception of 12h post infection in which there was a non-significant increase (Table 4).

IL-6 (*Il6*) is a cytokine released during inflammation and involved in the induction of hepcidin expression. Both infections, with *L. monocytogenes* and *S*. Typhimurium induced the expression of *Il6* in liver, although with different kinetics and to different degrees (Tables 3 and 4). While in animals infected with *L. monocytogenes*, *Il6* expression was induced in two waves, i.e. an early induction at 2h, followed by a decrease and another increase starting from 48h after infection (Table 3), during infection with *S*. Typhimurium, *Il6* expression undergoes an increase until 24h post infection, with the highest levels observed for this time-point, when compared with control animals (Table 4).

The expression of transferrin (*Tf*), the plasma iron transporter, also showed statistically significant alterations during infection with *L. monocytogenes* and *S*. Typhimurium, most notably at 48h and 72 hours post-infection (5 fold and 2 fold increases, for *L. monocytogenes* and *S*. Typhimurium respectively). Additionally, during *S*. Typhimurium infection, animals presented significant alterations in *Tf* expression at early time-points, with a slight but significant increase at 2h post infection and a 4-fold decrease at 6h after infection when compared with control non-infected mice.

Ferritin is an intracellular iron storage protein, composed of two different peptides, H-ferritin (*Fth1*) and L- ferritin (*Ftl*). We measured the expression of both *Fth1* and *Ftl* in liver during bacterial infections. The expression of *Ftl* was not significantly affected by any of the infectious agents used (Tables 3 and 4). Regarding *Fth1*, its expression did not vary significantly during infection with *L. monocytogenes* except for a 2-fold decrease at 72h post-infection. During infection with *S*. Typhimurium, *Fth1* expression had an earlier decrease at 12h after infection followed by significant increases at 24h and 72h after infection, comparing with control mice.

The expression of the cellular iron exporter, the ferroportin (*Fpn1*), was also measured. Both infectious agents induced the same response, i.e. a decrease of ferroportin expression, but with different kinetics. During *L. monocytogenes* infection, *Fpn1* expression presented significant decreases from 24h until 96h after infection, comparing to reference animals. In infection with *S*. Typhimurium, the expression of *Fpn1* significantly decreased for the first 6h post infection and at 48h and 72h after infection, when compared with control group.

Table 3: Alterations in the liver expression of genes involved in iron metabolism during *L. monocytogenes* infection.

	2h	6h	12h	24h	48h	72h	96h
Hamp1	1.20 ± 0.63	2.65 ± 1.14**	0.98 ± 0.15	1.37 ± 0.44	2.2 ± 1.48*	2.91 ±1.03***	2.35 ± 1.9
Il6	25.9 ± 19.4**	1.94 ± 0.92*	1.55 ± 1.18	5.7±3.4**	18.3±7.6***	9.9±7.7***	8.14 ± 3.3***
Tf	1.35 ± 0.49	1.63 ± 0.17	0.97 ± 0.09	1.71 ± 0.27	5.30 ±3.60***	4.67 ±1.40***	1.46 ± 1.29
Fth1	0.97 ± 0.31	0.77 ± 0.05	1.26 ± 0.33	0.73 ± 0.14	0.70 ± 0.19	0.45 ± 0.32 *	0.82 ± 0.47
Ftl	1.07 ± 0.31	1.16 ± 0.15	1.41 ± 0.11	0.75 ± 0.12	3.7 ± 5.56	0.98 ± 0.54	0.96 ± 0.63
Fpn1	1.05 ± 0.34	1.59 ± 0.42	0.67 ± 0.23	0.26 ± 0.1*	0.15 ± 0.03**	0.18 ± 0.04**	0.26 ± 0.09*

C57BL6 mice were infected with *L. monocytogenes* or injected with an equivalent volume of saline solution. At the indicated time-points after infection, mice were sacrificed and genes involved in iron metabolism were quantified in liver lysates by RT PCR. The table shows the mean ± SD of the fold increase of expression in infected versus control animals, from 3 to 5 animals per group. Statistical significance was obtained by one-way ANOVA followed Bonferroni post hoc test. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4: Alterations in the liver expression of genes involved in iron metabolism during *S.* Typhimurium infection.

	2h	6h	12h	24h	48h	72h
Hamp1	7,64 ± 2,36***	3.81 ± 1.06***	1.43 ± 0.37	4.35 ± 0.73***	6.45 ± 0.49***	13.78 ±9.23***
Il6	1.87 ± 0.71	3.08 ± 0.49**	4,85 ± 5,54*	31 ± 14,54***	3.19 ± 3.37*	2.88 ± 2.85*
Tf	1.64 ± 0.05*	0.27 ± 0.05*	0.75 ± 0.30	1.61 ± 0.77	2.01 ± 0.88**	2.28 ± 0.85**
Fth1	1.31 ± 0.44	1.06 ± 0.43	0.33 ± 0.244*	2.07 ± 1.54*	1.72 ± 1.422	3.86 ± 2.47***
Ftl	0.90 ± 0.27	0.20 ± 0.21	0.69 ± 0.53	1.07 ± 0.91	1.00 ± 0.09	0.61 ± 1.39
Fpn1	0.21 ± 0.17**	0.05 ± 0.05***	0.57 ± 0.39	1.01 ± 0.60	0.23 ± 0.09**	0.21 ± 0.07**

C57BL6 mice were infected with *S.* Typhimurium or injected with an equivalent volume of saline solution. At the indicated time-points after infection, mice were sacrificed and genes involved in iron metabolism were quantified in liver lysates by RT PCR. The table shows the mean ± SD of the fold increase of expression in infected versus control animals, from 3 to 5 animals per group. Statistical significance was obtained by one-way ANOVA followed Bonferroni post hoc test. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5. Infection with *L. monocytogenes* and *S.* Typhimurium leads to alterations in non-heme iron concentration in the liver

Liver is the main organ involved in iron storage. Figure 13 shows non-heme iron concentration in the liver during bacterial infection with *L. monocytogenes* (Figure 13A) and *S.* Typhimurium (Figure 13B) when compared to control non-infected mice. The concentration of non-heme iron in the liver tended to be higher in animals infected with both *L. monocytogenes* and *S.* Typhimurium, when compared to control animals (Figure 13A and 13B).

Animals infected with *L. monocytogenes* presented significant increases for 12h, 48h, 72h and 96h after infection. The highest levels of non-heme iron in liver were observed at 12h post infection with infected mice presenting two times the amount of iron detected in control mice (Figure 13A). On the other hand, animals infected with *S.* Typhimurium showed significant increases starting from 12h after infection until the end of experiment with the highest amount of non-heme iron in liver observed at 24h post infection. At this time-point, the infected animals presented about 2.5 times more iron than controls (Figure 13B).

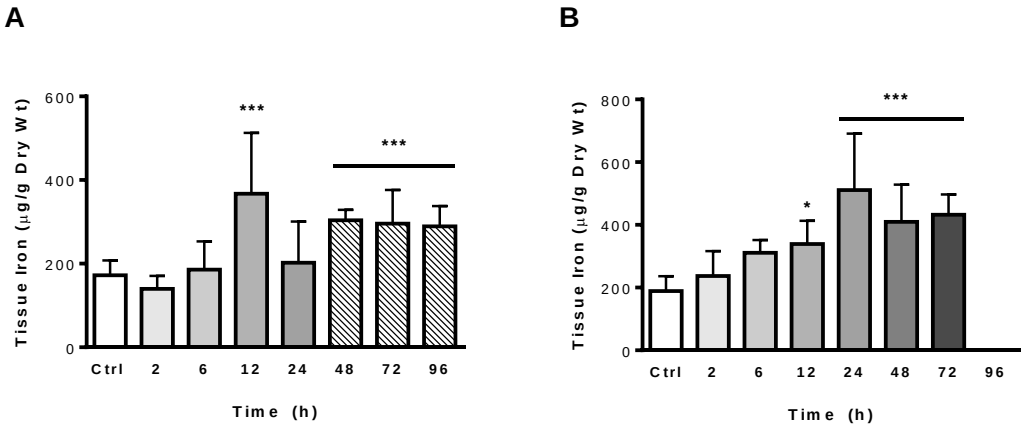

Figure 13: Non-heme iron concentration in the liver during bacterial infection with *L. monocytogenes* and *S.* Typhimurium. Bacterial infection with *L. monocytogenes* and *S*. Typhimurium induces alterations in iron content in the liver. Graphs depict mean ± SD of 3 to 5 animals. Statistical significance was evaluated by one-way ANOVA followed by Bonferroni post hoc test. *p <0.05 and ***p <0.001 when compared with control animals. Pattern bars represent data from a non-simultaneous experiment.

6. Infection with *L. monocytogenes* and *S.* Typhimurium does not cause significant alterations of iron distribution in the liver but it leads to changes in tissue structure.

Given the differences observed in liver iron concentration during infection with *L. monocytogenes* and *S*. Typhimurium, we decided to evaluate whether there were also alterations in liver iron distribution. Liver sections were analysed after Perls Prussian blue staining. However, using this technique we did not detect significant alterations in iron distribution in the liver (data not shown). Nevertheless, this procedure allowed to verify that the structure of liver in infected animals was modified after 72h of infection and, therefore, hematoxylin-eosin staining (H&E) was performed for time-points after 72h of infection.

The H&E protocol showed that non-infected mice present a normal liver architecture with well-defined hepatocytes containing central nuclei (Figure 14A and 17A). On the other hand, in mice infected with *L. monocytogenes* and *S*. Typhimurium it is possible to observe structural alterations in the liver when compared with control animals, including the loss of cell integrity and cell borders, being the visualization of nuclei more difficult. These conditions suggest that cells can be in cellular death. Furthermore, in both infections with *L. monocytogenes* and *S.* Typhimurium, mononuclear cells infiltrates are detected (Figure 14B, C and 17B, C). In some of these infiltrates it is possible observe what appear to be cells with phagocytosed material, presumably apoptotic bodies (Figure 16). In infections with both pathogens, acidophilic areas are identified. This alteration is evidenced by dark pink areas (Figure 14D and 17D) and it is not present in control non-infected group. Histopathological

alterations observed during infections are more visible and frequent during infection with *S*. Typhimurium than in infection with *L. monocytogenes.*

Finally, liver of mice infected with *S*. Typhimurium also presented the extravasation of erythrocytes to neighbours areas of blood vessel (Figure 17D), a feature that is not common in healthy animals.

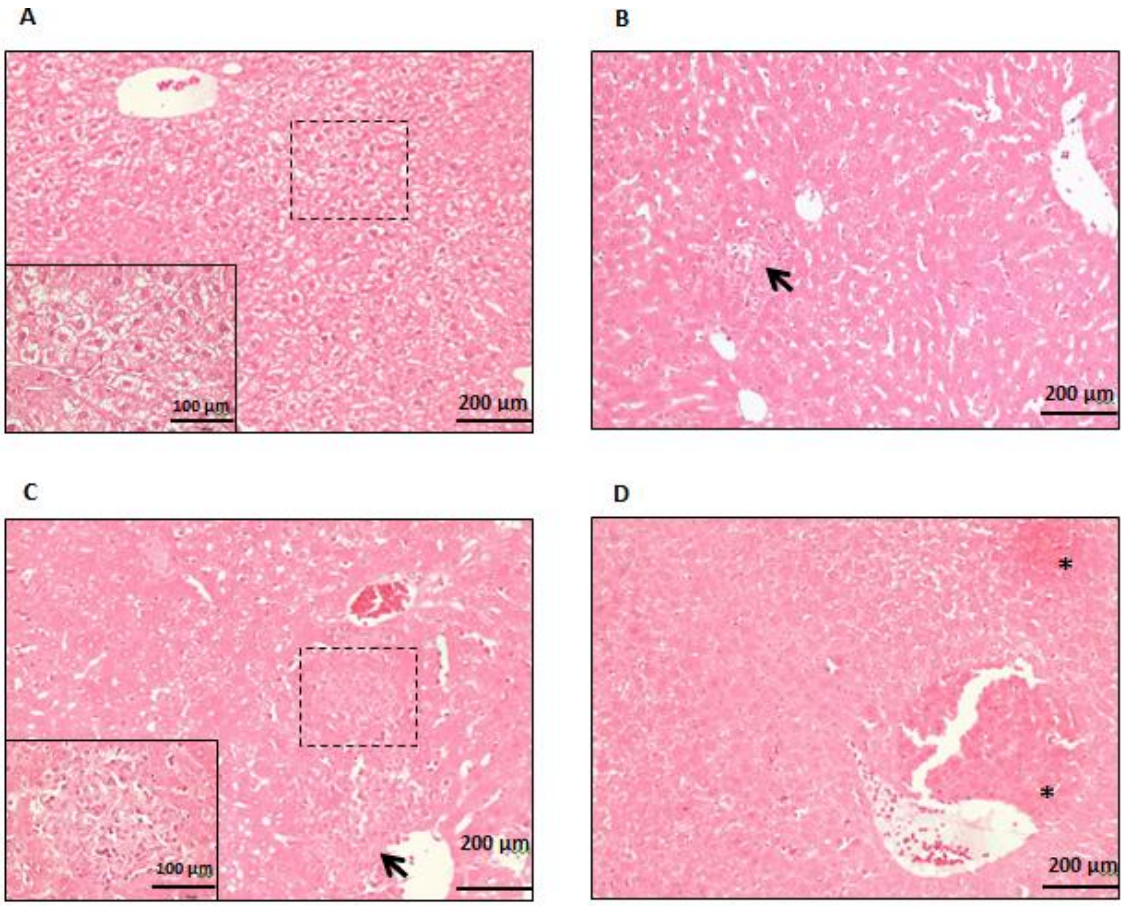

Figure 14: Histopathological features observed during infection with *L. monocytogenes*. Liver samples were collected from control and infected mice for histological examination. Liver sections were analysed by H&E staining to investigate structural alterations during infection with *L. monocytogenes* (A) Control mice; (B) Infiltrate of mononuclear cells at 72h post-infection; (C) Infiltrate of mononuclear cells at 96h post-infection. (D) Acidophilic areas in the vicinity of a blood vessel at 96h after infection. Arrows indicate infiltrates of mononuclear cells. Stars indicate acidophilic areas. Pictures were obtained at 10x magnification. The dotted squares are detailed in the insets (40x magnification).

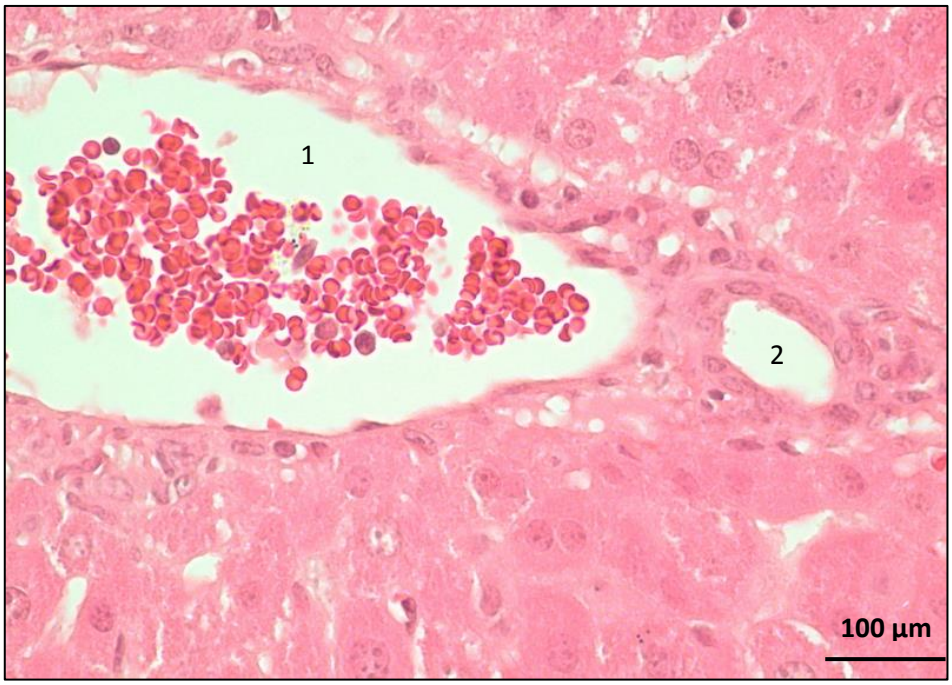

Figure 15: Portal Triad showing the portal vein with erythrocytes (1) and the bile duct (2) at 72h after infection with *L. monocytogenes*.

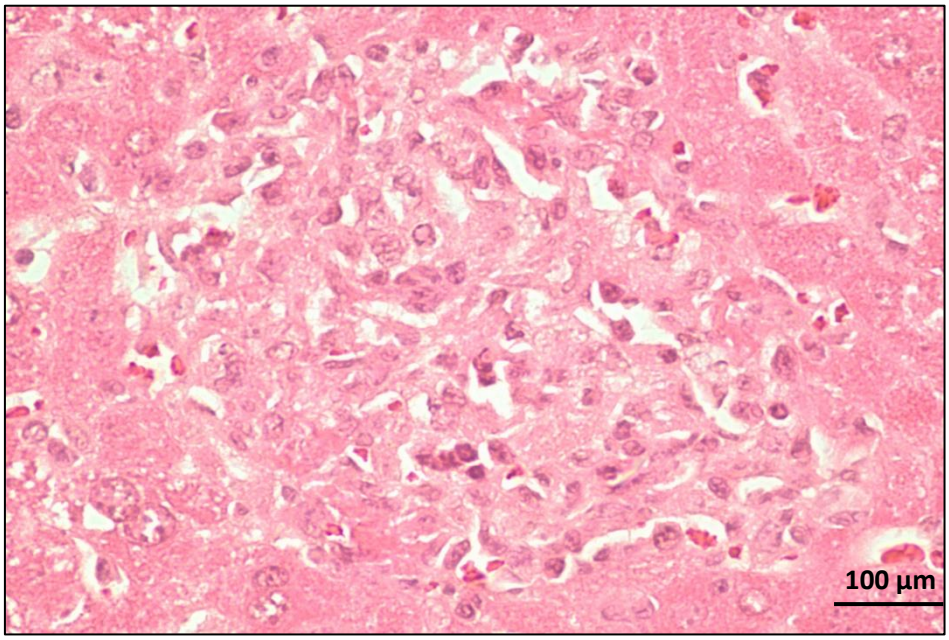

Figure 16: Infiltrate of mononuclear cells during infection with *L. monocytogenes*.

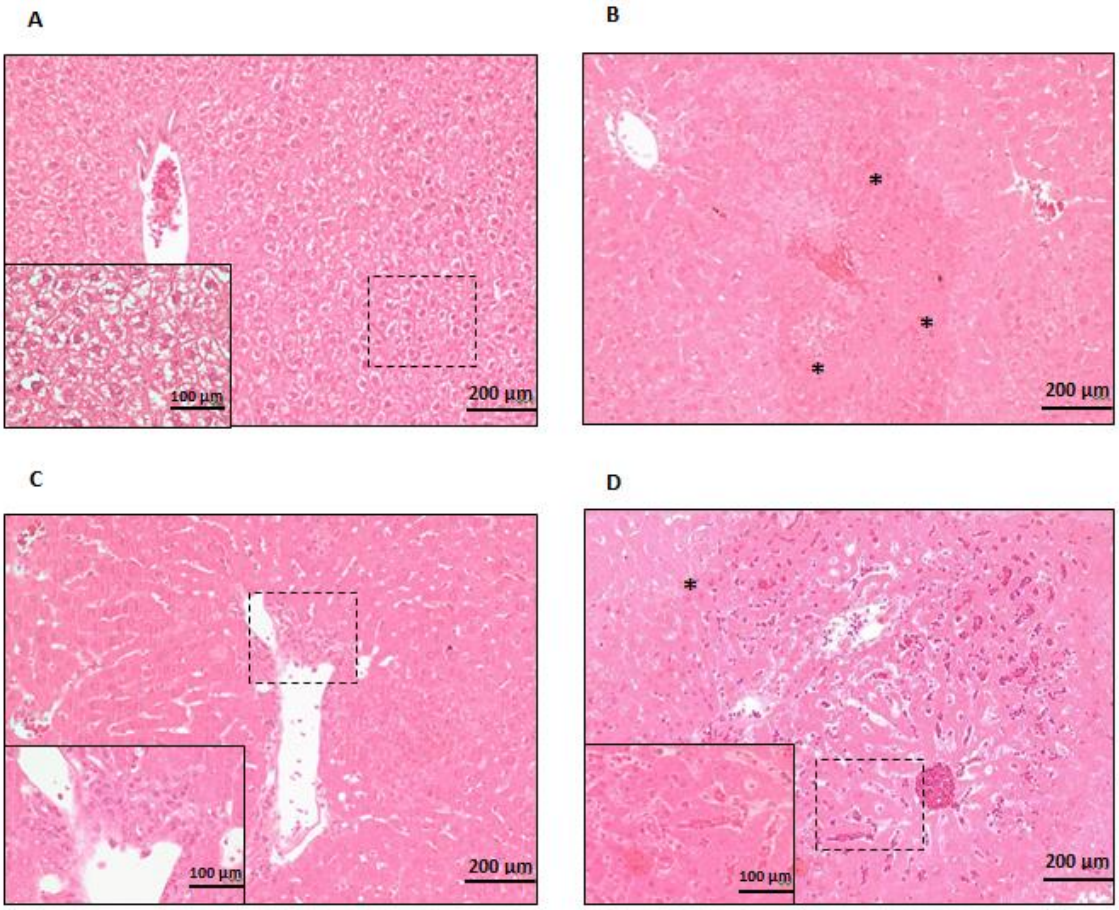

Figure 17: Histopathological features observed during infection with *S*. Typhimurium. Liver samples were collected from control and infected mice to histological examination. Liver sections were analysed by H&E staining to investigate structural alterations during infection with *S*. Typhimurium. (A) Control mice; (B) Acidophilic areas at 72h post-infection. (C) Infiltrate of mononuclear cells at 72h post-infection; (D) Extravasation of RBCs into neighbouring areas of blood vessel after 72h of infection. Stars indicate acidophilic areas. Pictures were obtained at 10x magnification. The dotted squares are detailed in the insets (40x magnification).

V. DISCUSSION

In this work, we were interested in comparing the iron metabolism in the host during infection with *Listeria monocytogenes*, a gram positive bacterium, and with *Salmonella* Typhimurium, a gram negative bacterium.

Iron, immunity and infection are intricately connected and its regulation is crucial for the mammal host survival. Iron is an abundant metal on earth and is vital for both mammals and invading microbes, which triggers the competition for this element during infection (Latunde-Dada 2009, Kortman et al. 2012, Nairz et al. 2014).

As previously said iron is an essential element for microbial growth and proliferation. Moreover, iron availability is frequently involved in the expression of virulence-associated properties in pathogenic bacteria. Thus, the deprivation of iron severely reduces the pathogenicity of *L. monocytogenes* and *S.* Typhimurium. Consequently, during bacterial infection, microbes use several mechanisms to obtain iron from the host (Kortman et al. 2012, Rodriguez et al. 2014).

On the other hand, the host also needs iron for several metabolic pathways, including oxygen sensing and transport, nuclei acid synthesis, erythropoiesis and immune responses. For this purpose, the host sequesters the iron in forms and locations that are less accessible to pathogens, thus reducing their proliferation (Rodriguez et al. 2014).

Given the central role played by the liver in iron metabolism, this organ was harvested from animals and used to perform different tests including the evaluation of bacterial load, non-heme iron determination, iron distribution in tissue, histopathological analyses and the evaluation of hepatic mRNA expression of iron-related genes.

Previous studies showed that after intravenous injection of *L. monocytogenes*, this bacterium is able to reach the liver and spleen in a few minutes. In these organs, bacteria are quickly cleared and phagocytized by resident and activated phagocytes, allowing the death of a large population of bacteria (Conlan 1996, Ramaswamy et al. 2007, Zenewicz and Shen 2007). The same is observed for *S*. Typhimurium (Mittrucker and Kaufmann 2000). In our results from bacterial load in liver, during infections with *L. monocytogenes* and *S*. Typhimurium (Figure 10), it is possible to note that there was a slight decrease in liver bacterial load during the first 2-6h after both infections, which illustrates the tentative of the host to fight the infections. When the infection is not controlled, bacteria are able to proliferate and grow (Mittrucker and Kaufmann 2000, Ramaswamy et al. 2007).

The first stages of *Salmonella* infection are normally completed within a few hours and are followed by a phase of several days, during which intracellular multiplication of bacteria

occurs and bacterial titers increase in liver. In mice, approximately 10^8 bacteria appears to be the critical load for survival, and if bacterial titers reach this threshold, the animal is no longer able to contain the infection and secondary bacteremia, endotoxic shock, and rapid death occurs (Mittrucker and Kaufmann 2000). Once again, our results are consistent with these previous findings, since from 6h after infection with *L. monocytogenes* and *S*. Typhimurium, bacteria grown and proliferated in liver, which is translated by an increase in liver bacterial load over time and the death of *S*. Typhimurium at 96h after infection with approximately 10^8 CFU/liver (Figure 10B). However, in *L. monocytogenes* infection, bacterial load in liver experienced a slight decrease at 96h after infection. These results indicate that, for the same experimental conditions, *S*. Typhimurium is a more severe and powerful pathogen than *L. monocytogenes*, being lethal to host at 96h after infection.

Our results revealed that mice infected with *L. monocytogenes* exhibited a mild anaemia during infection, expressed by a reduction of RBCs counts and haematocrit with the highest incidence at 96h after infection. These findings are consistent with previous results, in which listeriosis can be accompanied by anaemia (Mullarky et al. 2005). These results were also observed during infection with other microorganisms such as *M. avium* (Rodrigues et al. 2011). Anaemia stimulated by *L. monocytogenes* can result from decreased erythropoiesis. Under physiologic conditions, iron is recycled by reticulo-endothelial macrophages, maintaining a sufficient supply of iron for erythropoiesis. However, during infection this process is disrupted leading to an impaired delivery of iron for erythropoiesis (Silva-Gomes et al. 2013, Nairz et al. 2014).

In turn, *S*. Typhimurium infection does not seem to affect the RBCs quantity during entire experiment, except at 72h after infection when RBCs number slightly decreased, although not significantly. This condition may indicate that animals are likely anaemic. This assumption was strengthened by the haematocrit values that are in accordance with RBCs counts, presenting significant decreases at 72h after infection with *S*. Typhimurium.

During infection with *L. monocytogenes* and *S*. Typhimurium there was an alteration in the expression of genes involved in iron metabolism, including *Hamp1*, *Il6*, *Tf*, *Fth1*, *Ftl* and *Fpn1*. Hepcidin (*Hamp1*) plays a central role in the immune response to pathogens, operating as a liver-expressed antimicrobial peptide with activity against a wide range of bacteria (Wallace et al. 2011). During infection, macrophages are activated and produce numerous pro-inflammatory cytokines, including IL-6, via the JAK-STAT pathway, and a variety of chemokines that recruit cells of the immune system to the infection site (Silva-Gomes et al. 2013, Nairz et al. 2014). IL-6 induces the formation of hepcidin in the liver (Nemeth et al. 2004, Lee et al.

2005), blocking the iron export by ferroportin and causing hypoferremia (Wallace et al. 2011, Deschemin and Vaulont 2013, Lokken et al. 2014, Rodriguez et al. 2014).

Additionally, it is known that during initial stages of infection, cell wall components of gram negative bacteria, such as *S*. Typhimurium, including LPS and certain lipoproteins induce a massive inflammatory response in the surrounding tissue, by the release of pro-inflammatory cytokines, most prominently IL-6, which, in turn, induce the expression of hepcidin (Mittrucker and Kaufmann 2000, Deschemin and Vaulont 2013, Nairz et al. 2014, Guida et al. 2015).

Our results showed that the expression of *Hamp1* was increased in both infections with *L. monocytogenes* and *S*. Typhimurium. However, *Hamp1* was much more expressed throughout the experiment with *S*. Typhimurium comparatively with the same time points of *L. monocytogenes* infection (Table 3 and 4). Additionally, *Hamp1* expression also showed a statistically significant increase earlier in infection with *S*. Typhimurium (2h after infection), while in infection with *L. monocytogenes*, *Hamp1* expression only had significant increases for 6h and 72h after infection (Table 3). These data may possibly be justified by the positive action of LPS, present in the outer membrane of *S*. Typhimurium, on hepcidin expression.

It is known that hepcidin expression is induced by IL-6 during inflammation. To study the correlation between these two molecules, the hepatic mRNA levels of the pro-inflammatory cytokine IL-6 were also measured in control and infected mice. However, our results showed that the direct relation between the expression levels of *Il6* and *Hamp1* is not always detected, as observed at 6h and 12h after infection with *L. monocytogenes* (Table 3). These findings may indicate that increased expression of *Il6* alone is not enough for the activation of hepcidin and that *Hamp1* levels can be controlled by other mechanisms besides the cytokine IL-6. Indeed, Fournier and their collaborators observed that activin B has a crucial role in the induction of hepcidin by inflammation, due to its effects on BMP signalling with Smad 1/5/8 phosphorylation. These investigators also showed that induction of activin B and hepcidin by LPS occurs independently of IL-6, which can explain the increased *Hamp1* expression although *Il6* expression are decreased (Besson-Fournier et al. 2012).

Furthermore, recent data revealed an additional role of transferrin as an up regulator of hepcidin (Gkouvatsos et al. 2012). In fact, in Table 3, during *L. monocytogenes* infection, the time-points where the *Tf* expression was higher (48h and 72h post-infection) were equivalent with time-points where the *Hamp1* expression was also high.

On the other hand, it is known that the induction of hepcidin expression by inflammatory stimuli may explain the hypoferremia associated with anaemia through the capacity of hepcidin to bind ferroportin, the only known cellular iron exporter. This binding

leads to the internalization and degradation of ferroportin and, consequently, cellular iron retention with decreased iron export (Nemeth et al. 2004, Silva-Gomes et al. 2013, Lokken et al. 2014, Rodriguez et al. 2014). Reduced iron export from macrophages that recycle iron from senescent red blood cells induces hypoferremia (Guida et al. 2015). This mechanism reduces the serum iron content to about 30% of its normal level and it is considered a primary defensive mechanism of host, restricting the iron availability to invading pathogens (Wallace et al. 2011, Deschemin and Vaulont 2013, Kautz et al. 2014, Lokken et al. 2014, Rodriguez et al. 2014). Furthermore, sequestration of iron by ferritin inside the cells during infection and inflammation also leads to hypoferremia (Latunde-Dada 2009).

Although effects of hepcidin on ferroportin occur at protein level, hepatic mRNA levels were also measured. In our results, the decrease of hepatic mRNA levels of ferroportin (*Fpn1*) followed the increase of hepatic mRNA levels of hepcidin (*Hamp1*) in infected mice during *L. monocytogenes* and *S*. Typhimurium infections. A recent study evidenced an acute inflammatory condition in which TLR2/6 pathway induces hypoferremia by triggering decreases in ferroportin mRNA expression in liver of mice without modifying hepcidin expression (Guida et al. 2015). These results challenge the prevailing role of hepcidin in inflammatory hypoferremia and suggest that there is an alternative mechanism, which can be responsible for the hypoferremia during infection.

In turn, decreased expression of *Fpn1* should be accompanied by decreased levels of iron in serum, since iron is accumulated within cells. Although our results indicate decreased expression of *Fpn1* in time-points between 24h and 96h after infection with *L. monocytogenes* (Table 3), this decrease was only accompanied by significantly decreased levels of iron in serum for 24h and 48h after infection (Figure 12A). In contrast, the *Fpn1* expression presented a significant decrease at 96h after infection with *L. monocytogenes*. However, serum iron levels were significantly increased in this time-point. This condition can be explained by several ways: 1) Iron can be stored in other organs or other cells. In literature there are references that iron can be stored in duodenum (Zhang et al. 2011, Kong et al. 2013) and spleen (Kong et al. 2013) and to be released into the serum by ferroportin during iron starvation. We have also harvested spleen and duodenum samples for further analysis; 2) Increase of serum iron levels can happen as a compensatory mechanism, since iron levels were decreased in previous time points; 3) As *L. monocytogenes* is a facultative intracellular pathogen, the host can increase serum iron levels in order to make intracellular iron unavailable to the microorganism (Lokken et al. 2014).

In respect to *S*. Typhimurium infection, the *Fpn1* expression was significantly reduced at 2h, 6h, 48h and 72h after infection (Table 4) and this decrease was followed by decreased iron serum levels for 2h, 6h and 48h after infection. However, accordance with infection with *L. monocytogenes*, at the end of the experiment, in this case at 72h after infection, the serum iron levels also presented a slight increase (Figure 12E). The explanation for this condition can be the same presented above.

Our results also showed that, as expected, saturated transferrin levels (TSAT) were in accordance with serum iron levels for both infections with *L. monocytogenes* (Figure 12B) and *S.* Typhimurium (Figure 12F). Control animals also showed expected values, since it is known that under normal conditions about 20% to 40% of iron binding sites in transferrin are saturated (Murtagh et al. 2002, Gkouvatsos et al. 2012).

Additionatly, unsaturated iron binding capacity (UIBC) is a measurement of the unsaturated fraction of serum transferrin (Murtagh et al. 2002). It is expected that UIBC levels are elevated when TSAT levels are decreased. Our results are in accordance with this, since during infection with *L. monocytogenes,* TSAT presented their lower values for 48h and 72h after infection, which corresponds to the highest values of UIBC. On the other hand, during infection with *S.* Typhimurium, TSAT levels were reduced starting from 6h after infection, which was accompanied by increased UIBC levels.

Finally, total iron binding capacity (TIBC) values represent how well transferrin binds and carries iron in the blood. TIBC values tended to follow the UIBC values for both infections with *L. monocytogenes* and *S.* Typhimurium. The host increases the TIBC values in order to maximize the use of iron by transferrin.

Moreover, hypoferremia promotes anaemia and, thus, decreased numbers of RBCs and HCT are expected (Weiss 2009, Guida et al. 2015). However, in our experiments with *L. monocytogenes*, although there were evidences of anaemia at 96h after infection with decreased levels of RBCs and HCT (Figure 11A and Figure 11B), this reduction did not follow the serum iron levels which were elevated for this time-point. These results can be indicative that the iron taken up by reticulo-endothelial macrophages at 96h after infection with *L. monocytogenes* is not enough for erythropoiesis. Likewise, during infection with *S*. Typhimurium, serum iron levels were decreased for 6h and 12h after infection, but these decreases were not accompanied by significant decreases for RBCs and HCT for the same time points.

Plasma transferrin is a central player in iron metabolism, since protect from free iron toxicity by binding to iron (Hentze et al. 2010, Gkouvatsos et al. 2012). It is predominantly

synthesized in the human liver (Lambert et al. 2005, Gkouvatsos et al. 2012). Previous studies showed that, during inflammation, transferrin can act either as a negative or a positive acute phase protein in mammals (Schreiber et al. 1989, Ritchie et al. 1999).

During *L. monocytogenes* infection transferrin responded to bacterial infection significantly increasing its expression at 48h and 72h after infection. These results are consistent with the decreased serum iron levels for these time-points, since this reduction is, at least partially, mediated by transferrin. Thus, in liver of infected mice, transferrin seems to function as a positive acute phase protein.

In turn, during *S*. Typhimurium infection, the animals presented statistically significant increases of transferrin early in infection (2h post infection) and for the last 2 days of experiment (48h and 72h after infection). Inversely, at 6h after infection with *S*. Typhimurium, the transferrin showed a significant reduction in their expression, which is surprising since from our data, the iron levels in liver in this time-point were similar to control animals (Figure 13B).

Ferritin, the iron storage protein, is synthesized when iron is available in order to store iron and reduce its availability for pathogens, whereas under iron deprivation conditions, ferritin synthesis is repressed. Up-regulation of ferritin is also observed in inflammatory conditions, being induced by exposure to LPS and pro-inflammatory cytokines (Torti and Torti 2002). Moreover, Nairz and collaborators also showed that the infection with *S*. Typhimurium leads to a marked increase of ferritin mRNA levels likely because it is a gram negative bacterium and has LPS in their wall that we have seen above that stimulates the ferritin expression (Nairz et al. 2008). Our results are in accordance with this, since in *L. monocytogenes* infection, the *Fth1* and *Ftl* expression did not appear to be greatly affected by infection. However, during *S.* Typhimurium infection, *Fth1* expression was increased for all time-points with the exception of 12h post-infection with statistically significant increases for 24h and 72h after infection. This increased expression is consistent with the need to increase iron storage in order to make it unavailable for bacterial growth. Furthermore, the highest values for *Fth1* expression were in agreement with the highest levels of iron concentration in liver (Figure 13B).

Liver is the main organ involved in iron storage, storing about 200 mg of total iron (Stein et al. 2010). Non-heme iron quantity in liver was determined during bacterial infection. Results showed that iron amounts in liver were increased during bacterial infection with *L. monocytogenes* and *S*. Typhimurium against to control mice, which present approximately 200 µg/g dry weight. However, *L. monocytogenes* infection had a peak of iron quantity in liver

earlier (12h after infection) than *S*. Typhimurium infection (24h after infection). As the iron amounts in serum during *S*. Typhimurium infection started to decrease at 6h after infection, these results suggest that the liver is not the first organ to capture iron during infection with *S*. Typhimurium.

Our results obtained by Perl's Prussian blue staining showed that infection with *L. monocytogenes* and *S.* Typhimurium induce no significant alterations on liver distribution. These observations are consistent with those previously reported by our group for another microorganism, namely *M. avium* (Rodrigues et al. 2011).

However, Perls Prussian blue staining allowed the observation that the structure of liver in infected animals was altered at 72h post-infection. Thus, representative liver sections were stained with hematoxylin-eosin staining, a technique that allows the study of tissue morphology in detail. Our results confirm that infection with both *L. monocytogenes* and *S*. Typhimurium leads to alterations in tissue structure with loss of normal cell structure and well-defined cell borders, when compared with control animals. Nuclei are also more difficult to observe, which indicates degeneration of that cell. These cellular alterations suggest that during infection with these pathogens, the cellular integrity is loss. Previous studies showed that non-heme iron in liver which is initially sequestered in lysosomes can be released and taken up by mitochondria (Uchiyama et al. 2008). Increased levels of iron may lead to mitochondrial dysfunction, resulting in ROS production and its release into the cytosol. ROS are probably responsible for the cellular injury and death (Gao et al. 2010).

Other alteration observed in infected mice is based on the presence of mononuclear cells infiltrates (Figure 14C, D and Figure 17B,C), containing what appears to be phagocytosed material. This can indicate that the host was trying to fight the infection by immune system in which phagocytic cells, such as neutrophils and macrophages are attracted to infection site and phagocytize bacteria.

On the other hand, liver of mice infected with *L. monocytogenes* and *S*. Typhimurium presented acidophilic areas, evidenced by dark pink areas (Figure 14 D and Figure 7B,D). This contrast with surrounding areas is justified by the greatest attraction of eosin, an acid dye, to these areas, possibly due to the presence of more basic components. These histopathological alterations can indicate biochemical alterations in tissue due to pathology and infection. There are also the presence of damage tissue in infected mice which can also indicate that the tissue is more weakened due to the infection.

Finally, liver of mice infected with *S*. Typhimurium seemed to present an extravasation of red blood cells to neighbouring areas of central blood vessel (Figure 17D). These results can also denote the loss of tissue integrity with more vulnerable blood vessels.

The work developed in this thesis came to complement the previous findings obtained by the group, since the alterations on iron metabolism during the infection with other pathogens, such as *Mycobacterium avium*, was previously assessed (Rodrigues et al. 2011). Summing up, our data suggest that the alterations in iron metabolism differently occur depending on the bacterium, with *S*. Typhimurium causing earlier and more evident changes in serological and gene expression parameters. Answering the questions we established in the beginning of this work: depending on the bacterial infection, the alterations of iron metabolism in the host appear to be different and be related with the hepcidin levels, although, changes in hepcidin mRNA levels does not appear to be the unique mechanism involved in these alterations.

As future perspectives, the identification of specific mechanisms involved in iron homeostasis and the understanding of which pathways are involved in host-pathogen interactions may be promising strategies to discover new and significant therapeutic targets to deal with infections with *L. monocytogenes*, *S*. Typhimurium and other microorganisms. Furthermore, it is expected that investigators help to find new ways to deprive the pathogen from iron without altering normal iron metabolism in host.

VI. REFERENCES

Achard, M. E., K. W. Chen, M. J. Sweet, R. E. Watts, K. Schroder, M. A. Schembri and A. G. McEwan (2013). "An antioxidant role for catecholate siderophores in Salmonella." Biochem J **454**(3): 543-549.

Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen Recognition and Innate Immunity." Cell **124**(4): 783-801.

Alberts, B., J. Wilson and T. Hunt (2008). Molecular biology of the cell. New York, Garland Science.

Anderson, C. P., M. Shen, R. S. Eisenstein and E. A. Leibold (2012). "Mammalian iron metabolism and its control by iron regulatory proteins." Biochim Biophys Acta **1823**(9): 1468-1483.

Andrews, N. C. (2000). "Iron homeostasis: insights from genetics and animal models." Nat Rev Genet **1**(3): 208-217.

Andrews, N. C. and P. J. Schmidt (2007). "Iron homeostasis." Annu Rev Physiol **69**: 69-85.

Ashby, D. R., D. P. Gale, M. Busbridge, K. G. Murphy, N. D. Duncan, T. D. Cairns, D. H. Taube, S. R. Bloom, F. W. Tam, R. Chapman, P. H. Maxwell and P. Choi (2010). "Erythropoietin administration in humans causes a marked and prolonged reduction in circulating hepcidin." Haematologica **95**(3): 505-508.

Athman, R. and D. Philpott (2004). "Innate immunity via Toll-like receptors and Nod proteins." Curr Opin Microbiol **7**(1): 25-32.

Basset, C., J. Holton, R. O'Mahony and I. Roitt (2003). "Innate immunity and pathogen-host interaction." Vaccine **21 Suppl 2**: S12-23.

Berche, P. (2005). "[Pathophysiology and epidemiology of listeriosis]." Bull Acad Natl Med **189**(3): 507-516; discussion 516-521.

Besson-Fournier, C., C. Latour, L. Kautz, J. Bertrand, T. Ganz, M. P. Roth and H. Coppin (2012). "Induction of activin B by inflammatory stimuli up-regulates expression of the iron-regulatory peptide hepcidin through Smad1/5/8 signaling." Blood **120**(2): 431-439.

Beutler, B. (2004). "Innate immunity: an overview." Mol Immunol **40**(12): 845-859.

Bjorkqvist, J., A. Jamsa and T. Renne (2013). "Plasma kallikrein: the bradykinin-producing enzyme." Thromb Haemost **110**(3): 399-407.

Bonilla, F. A. and H. C. Oettgen (2010). "Adaptive immunity." J Allergy Clin Immunol **125**(2 Suppl 2): S33-40.

Bourhis, L. L. and C. Werts (2007). "Role of Nods in bacterial infection." Microbes Infect **9**(5): 629-636.

Braun, V. (2001). "Iron uptake mechanisms and their regulation in pathogenic bacteria." Int J Med Microbiol **291**(2): 67-79.

Brown, J. S. and D. W. Holden (2002). "Iron acquisition by Gram-positive bacterial pathogens." Microbes Infect **4**(11): 1149-1156.

Broz, P., M. B. Ohlson and D. M. Monack (2012). "Innate immune response to Salmonella typhimurium, a model enteric pathogen." Gut Microbes **3**(2): 62-70.

Cassat, J. E. and E. P. Skaar (2013). "Iron in infection and immunity." Cell Host Microbe **13**(5): 509-519.

Chaplin, D. D. (2006). "1. Overview of the human immune response." J Allergy Clin Immunol **117**(2 Suppl Mini-Primer): S430-435.

Charles, J. F., M. B. Humphrey, X. Zhao, E. Quarles, M. C. Nakamura, A. Aderem, W. E. Seaman and K. D. Smith (2008). "The innate immune response to Salmonella enterica serovar Typhimurium by macrophages is dependent on TREM2-DAP12." Infect Immun **76**(6): 2439-2447.

Cherayil, B. J. (2011). "The role of iron in the immune response to bacterial infection." Immunol Res **50**(1): 1-9.

Chlosta, S., D. S. Fishman, L. Harrington, E. E. Johnson, M. D. Knutson, M. Wessling-Resnick and B. J. Cherayil (2006). "The iron efflux protein ferroportin regulates the intracellular growth of Salmonella enterica." Infect Immun **74**(5): 3065-3067.

Claes, A. K., N. Steck, D. Schultz, U. Zahringer, S. Lipinski, P. Rosenstiel, K. Geddes, D. J. Philpott, H. Heine and G. A. Grassl (2014). "Salmonella enterica serovar Typhimurium DeltamsbB triggers exacerbated inflammation in Nod2 deficient mice." PLoS One **9**(11): e113645.

Conlan, J. W. (1996). "Early pathogenesis of Listeria monocytogenes infection in the mouse spleen." J Med Microbiol **44**(4): 295-302.

Coombes, B. K., Y. Valdez and B. B. Finlay (2004). "Evasive maneuvers by secreted bacterial proteins to avoid innate immune responses." Curr Biol **14**(19): R856-867.

Creagh, E. M. and L. A. O'Neill (2006). "TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity." Trends Immunol **27**(8): 352-357.

Crouch, M. L., M. Castor, J. E. Karlinsey, T. Kalhorn and F. C. Fang (2008). "Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of Salmonella enterica serovar Typhimurium." Mol Microbiol **67**(5): 971-983.

De Domenico, I., D. McVey Ward and J. Kaplan (2008). "Regulation of iron acquisition and storage: consequences for iron-linked disorders." Nat Rev Mol Cell Biol **9**(1): 72-81.

Deschemin, J. C. and S. Vaulont (2013). "Role of hepcidin in the setting of hypoferremia during acute inflammation." PLoS One **8**(4): e61050.

Doherty, C. P. (2007). "Host-pathogen interactions: the role of iron." J Nutr **137**(5): 1341-1344.

Dussurget, O., E. Dumas, C. Archambaud, I. Chafsey, C. Chambon, M. Hebraud and P. Cossart (2005). "Listeria monocytogenes ferritin protects against multiple stresses and is required for virulence." FEMS Microbiol Lett **250**(2): 253-261.

Ellermeier, J. R. and J. M. Slauch (2008). "Fur regulates expression of the Salmonella pathogenicity island 1 type III secretion system through HilD." J Bacteriol **190**(2): 476-486.

Evstatiev, R. and C. Gasche (2012). "Iron sensing and signalling." Gut **61**(6): 933-952.

Flo, T. H., K. D. Smith, S. Sato, D. J. Rodriguez, M. A. Holmes, R. K. Strong, S. Akira and A. Aderem (2004). "Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron." Nature **432**(7019): 917-921.

Frawley, E. R. and F. C. Fang (2014). "The ins and outs of bacterial iron metabolism." Mol Microbiol **93**(4): 609-616.

Ganz, T. (2011). Hepcidin and iron regulation, 10 years later.

Ganz, T. and E. Nemeth (2012). "Hepcidin and iron homeostasis." Biochim Biophys Acta **1823**(9): 1434-1443.

Gao, X., M. Qian, J. L. Campian, J. Marshall, Z. Zhou, A. M. Roberts, Y. J. Kang, S. D. Prabhu, X. F. Sun and J. W. Eaton (2010). "Mitochondrial dysfunction may explain the cardiomyopathy of chronic iron overload." Free Radic Biol Med **49**(3): 401-407.

Giacomini, E., E. Iona, L. Ferroni, M. Miettinen, L. Fattorini, G. Orefici, I. Julkunen and E. M. Coccia (2001). "Infection of human macrophages and dendritic cells with Mycobacterium tuberculosis induces a differential cytokine gene expression that modulates T cell response." J Immunol **166**(12): 7033-7041.

Gkouvatsos, K., G. Papanikolaou and K. Pantopoulos (2012). "Regulation of iron transport and the role of transferrin." Biochim Biophys Acta **1820**(3): 188-202.

Goldfine, H. and H. Shen (2007). "Listeria monocytogenes: Pathogenesis and Host Response." Springer.

Greenberg, S. and S. Grinstein (2002). "Phagocytosis and innate immunity." Curr Opin Immunol **14**(1): 136-145.

Guida, C., S. Altamura, F. A. Klein, B. Galy, M. Boutros, A. J. Ulmer, M. W. Hentze and M. U. Muckenthaler (2015). "A novel inflammatory pathway mediating rapid hepcidin-independent hypoferremia." Blood **125**(14): 2265-2275.

Hantke, K., G. Nicholson, W. Rabsch and G. Winkelmann (2003). "Salmochelins, siderophores of Salmonella enterica and uropathogenic Escherichia coli strains, are recognized by the outer membrane receptor IroN." Proc Natl Acad Sci U S A **100**(7): 3677-3682.

Hentze, M. W., M. U. Muckenthaler, B. Galy and C. Camaschella (2010). "Two to tango: regulation of Mammalian iron metabolism." Cell **142**(1): 24-38.

Holden, V. I., S. Lenio, R. Kuick, S. K. Ramakrishnan, Y. M. Shah and M. A. Bachman (2014). "Bacterial siderophores that evade or overwhelm lipocalin 2 induce hypoxia inducible factor 1alpha and proinflammatory cytokine secretion in cultured respiratory epithelial cells." Infect Immun **82**(9): 3826-3836.

Hornef, M. W., M. J. Wick, M. Rhen and S. Normark (2002). "Bacterial strategies for overcoming host innate and adaptive immune responses." Nat Immunol **3**(11): 1033-1040.

Ibarra, J. A. and O. Steele-Mortimer (2009). "Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival." Cell Microbiol **11**(11): 1579-1586.

Janakiraman, A. and J. M. Slauch (2000). "The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of Salmonella typhimurium." Mol Microbiol **35**(5): 1146-1155.

Jeon, J., H. Kim, J. Yun, S. Ryu, E. A. Groisman and D. Shin (2008). "RstA-promoted expression of the ferrous iron transporter FeoB under iron-replete conditions enhances Fur activity in Salmonella enterica." J Bacteriol **190**(22): 7326-7334.

Jin, B., S. M. Newton, Y. Shao, X. Jiang, A. Charbit and P. E. Klebba (2006). "Iron acquisition systems for ferric hydroxamates, haemin and haemoglobin in Listeria monocytogenes." Mol Microbiol **59**(4): 1185-1198.

Kautz, L., G. Jung, E. Nemeth and T. Ganz (2014). "Erythroferrone contributes to recovery from anemia of inflammation." Blood **124**(16): 2569-2574.

Kindt, T. J., R. A. Goldsby, B. A. Osborne and J. Kuby (2007). Kuby immunology. New York, W.H. Freeman.

Klebba, P. E., A. Charbit, Q. B. Xiao, X. X. Jiang and S. M. Newton (2012). "Mechanisms of iron and haem transport by Listeria monocytogenes." Molecular Membrane Biology **29**(3-4): 69-86.

Knutson, M. D., M. Oukka, L. M. Koss, F. Aydemir and M. Wessling-Resnick (2005). "Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1 overexpression and down-regulated by hepcidin." Proc Natl Acad Sci U S A **102**(5): 1324-1328.

Kong, W. N., Y. H. Lei and Y. Z. Chang (2013). "The regulation of iron metabolism in the mononuclear phagocyte system." Expert Rev Hematol **6**(4): 411-418.

Kortman, G. A., A. Boleij, D. W. Swinkels and H. Tjalsma (2012). "Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface." PLoS One **7**(1): e29968.

Lambert, L. A., H. Perri, P. J. Halbrooks and A. B. Mason (2005). "Evolution of the transferrin family: conservation of residues associated with iron and anion binding." Comp Biochem Physiol B Biochem Mol Biol **142**(2): 129-141.

Latunde-Dada, G. O. (2009). "Iron metabolism: microbes, mouse, and man." Bioessays **31**(12): 1309-1317.

Lechowicz, J. and A. Krawczyk-Balska (2015). "An update on the transport and metabolism of iron in Listeria monocytogenes: the role of proteins involved in pathogenicity." Biometals.

Lecuit, M. (2005). "Understanding how Listeria monocytogenes targets and crosses host barriers." Clin Microbiol Infect **11**(6): 430-436.

Ledala, N., M. Sengupta, A. Muthaiyan, B. J. Wilkinson and R. K. Jayaswal (2010). "Transcriptomic response of Listeria monocytogenes to iron limitation and Fur mutation." Appl Environ Microbiol **76**(2): 406-416.

Lee, P., H. Peng, T. Gelbart, L. Wang and E. Beutler (2005). "Regulation of hepcidin transcription by interleukin-1 and interleukin-6." Proc Natl Acad Sci U S A **102**(6): 1906-1910.

Lin, H., M. A. Fischbach, D. R. Liu and C. T. Walsh (2005). "In vitro characterization of salmochelin and enterobactin trilactone hydrolases IroD, IroE, and Fes." J Am Chem Soc **127**(31): 11075-11084.

Lokken, K. L., R. M. Tsolis and A. J. Baumler (2014). "Hypoferremia of infection: a double-edged sword?" Nat Med **20**(4): 335-337.

Ludwiczek, S., E. Aigner, I. Theurl and G. Weiss (2003). "Cytokine-mediated regulation of iron transport in human monocytic cells." Blood **101**(10): 4148-4154.

Luo, M., H. Lin, M. A. Fischbach, D. R. Liu, C. T. Walsh and J. T. Groves (2006). "Enzymatic tailoring of enterobactin alters membrane partitioning and iron acquisition." ACS Chem Biol **1**(1): 29-32.

Male, D., Brostoff, Roth and Roitt (2006). "Immunology " Elsevier Health Sciences **7th edition**: 552.

Mariathasan, S., K. Newton, D. M. Monack, D. Vucic, D. M. French, W. P. Lee, M. Roose-Girma, S. Erickson and V. M. Dixit (2004). "Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf." Nature **430**(6996): 213-218.

Mastroeni, P. (2002). "Immunity to systemic Salmonella infections." Curr Mol Med **2**(4): 393-406.

McLaughlin, H. P., C. Hill and C. G. Gahan (2011). "The impact of iron on Listeria monocytogenes; inside and outside the host." Curr Opin Biotechnol **22**(2): 194-199.

Mittrucker, H. W. and S. H. Kaufmann (2000). "Immune response to infection with Salmonella typhimurium in mice." J Leukoc Biol **67**(4): 457-463.

Mullarky, I. K., F. M. Szaba, K. N. Berggren, M. A. Parent, L. W. Kummer, W. Chen, L. L. Johnson and S. T. Smiley (2005). "Infection-stimulated fibrin deposition controls hemorrhage and limits hepatic bacterial growth during listeriosis." Infect Immun **73**(7): 3888-3895.

Muller, S. I., M. Valdebenito and K. Hantke (2009). "Salmochelin, the long-overlooked catecholate siderophore of Salmonella." Biometals **22**(4): 691-695.

Murtagh, L. J., M. Whiley, S. Wilson, H. Tran and M. L. Bassett (2002). "Unsaturated iron binding capacity and transferrin saturation are equally reliable in detection of HFE hemochromatosis." Am J Gastroenterol **97**(8): 2093-2099.

Nagy, T. A., S. M. Moreland and C. S. Detweiler (2014). "Salmonella acquires ferrous iron from haemophagocytic macrophages." Mol Microbiol **93**(6): 1314-1326.

Nairz, M., G. Fritsche, P. Brunner, H. Talasz, K. Hantke and G. Weiss (2008). "Interferon-gamma limits the availability of iron for intramacrophage Salmonella typhimurium." Eur J Immunol **38**(7): 1923-1936.

Nairz, M., D. Haschka, E. Demetz and G. Weiss (2014). "Iron at the interface of immunity and infection." Front Pharmacol **5**: 152.

Nairz, M., I. Theurl, S. Ludwiczek, M. Theurl, S. M. Mair, G. Fritsche and G. Weiss (2007). "The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular Salmonella typhimurium." Cell Microbiol **9**(9): 2126-2140.

Nemeth, E., S. Rivera, V. Gabayan, C. Keller, S. Taudorf, B. K. Pedersen and T. Ganz (2004). "IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin." J Clin Invest **113**(9): 1271-1276.

Nemeth, E., M. S. Tuttle, J. Powelson, M. B. Vaughn, A. Donovan, D. M. Ward, T. Ganz and J. Kaplan (2004). "Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization." Science **306**(5704): 2090-2093.

Pamer, E. G. (2004). "Immune responses to Listeria monocytogenes." Nat Rev Immunol **4**(10): 812-823.

Pan, X., B. Tamilselvam, E. J. Hansen and S. Daefler (2010). "Modulation of iron homeostasis in macrophages by bacterial intracellular pathogens." BMC Microbiol **10**: 64.

Payne, S. M., and Rey, A.R. (2004). " Pathogenic Escherichia coli, Shigella and Salmonella." Iron Transport in Bacteria **ASM Press**: 199–218.

Philpott, D. J. and S. E. Girardin (2004). "The role of Toll-like receptors and Nod proteins in bacterial infection." Mol Immunol **41**(11): 1099-1108.

Ramaswamy, V., V. M. Cresence, J. S. Rejitha, M. U. Lekshmi, K. S. Dharsana, S. P. Prasad and H. M. Vijila (2007). "Listeria--review of epidemiology and pathogenesis." J Microbiol Immunol Infect **40**(1): 4-13.

Raymond, K. N., E. A. Dertz and S. S. Kim (2003). "Enterobactin: an archetype for microbial iron transport." Proc Natl Acad Sci U S A **100**(7): 3584-3588.

Richardson, W. M., C. P. Sodhi, A. Russo, R. H. Siggers, A. Afrazi, S. C. Gribar, M. D. Neal, S. Dai, T. Prindle, Jr., M. Branca, C. Ma, J. Ozolek and D. J. Hackam (2010). "Nucleotide-binding oligomerization domain-2 inhibits toll-like receptor-4 signaling in the intestinal epithelium." Gastroenterology **139**(3): 904-917, 917 e901-906.

Rishi, G., D. F. Wallace and V. N. Subramaniam (2015). "Hepcidin: regulation of the master iron regulator." Biosci Rep **35**(3).

Ritchie, R. F., G. E. Palomaki, L. M. Neveux, O. Navolotskaia, T. B. Ledue and W. Y. Craig (1999). "Reference distributions for the negative acute-phase serum proteins, albumin, transferrin and transthyretin: a practical, simple and clinically relevant approach in a large cohort." J Clin Lab Anal **13**(6): 273-279.

Rodrigues, P. N., S. S. Gomes, J. V. Neves, S. Gomes-Pereira, M. Correia-Neves, C. Nunes-Alves, J. Stolte, M. Sanchez, R. Appelberg, M. U. Muckenthaler and M. S. Gomes (2011). "Mycobacteria-induced anaemia revisited: a molecular approach reveals the involvement of NRAMP1 and lipocalin-2, but not of hepcidin." Immunobiology **216**(10): 1127-1134.

Rodriguez, R., C. L. Jung, V. Gabayan, J. C. Deng, T. Ganz, E. Nemeth and Y. Bulut (2014). "Hepcidin induction by pathogens and pathogen-derived molecules is strongly dependent on interleukin-6." Infect Immun **82**(2): 745-752.

Ruckdeschel, K., O. Mannel and P. Schrottner (2002). "Divergence of apoptosis-inducing and preventing signals in bacteria-faced macrophages through myeloid differentiation factor 88 and IL-1 receptor-associated kinase members." J Immunol **168**(9): 4601-4611.

Ryan, K. J., C. G. Ray and J. C. Sherris (2010). Sherris medical microbiology. New York, McGraw Hill Medical.

Sasaki, Y., M. Noguchi-Sasaki, H. Yasuno, K. Yorozu and Y. Shimonaka (2012). "Erythropoietin stimulation decreases hepcidin expression through hematopoietic activity on bone marrow cells in mice." Int J Hematol **96**(6): 692-700.

Schaible, U. E. and S. H. Kaufmann (2004). "Iron and microbial infection." Nat Rev Microbiol **2**(12): 946-953.

Schreiber, G., A. Tsykin, A. R. Aldred, T. Thomas, W. P. Fung, P. W. Dickson, T. Cole, H. Birch, F. A. De Jong and J. Milland (1989). "The acute phase response in the rodent." Ann N Y Acad Sci **557**: 61-85; discussion 85-66.

Silva-Gomes, S., S. Vale-Costa, R. Appelberg and M. S. Gomes (2013). "Iron in intracellular infection: to provide or to deprive?" Front Cell Infect Microbiol **3**: 96.

Singh, B., S. Arora, P. Agrawal and S. K. Gupta (2011). "Hepcidin: A novel peptide hormone regulating iron metabolism." Clinica Chimica Acta **412**(11–12): 823-830.

Singh, B., S. Arora, P. Agrawal and S. K. Gupta (2011). "Hepcidin: a novel peptide hormone regulating iron metabolism." Clin Chim Acta **412**(11-12): 823-830.

Skaar, E. P. (2010). "The Battle for Iron between Bacterial Pathogens and Their Vertebrate Hosts." PLoS Pathog **6**(8): e1000949.

Sotolongo, J., J. Ruiz and M. Fukata (2012). "The Role of Innate Immunity in the Host Defense Against Intestinal Bacterial Pathogens." Current Infectious Disease Reports **14**(1): 15-23.

Stein, J., F. Hartmann and A. U. Dignass (2010). "Diagnosis and management of iron deficiency anemia in patients with IBD." Nat Rev Gastroenterol Hepatol **7**(11): 599-610.

Takeda, K. and S. Akira (2005). "Toll-like receptors in innate immunity." Int Immunol **17**(1): 1-14.

Tanno, T., N. V. Bhanu, P. A. Oneal, S. H. Goh, P. Staker, Y. T. Lee, J. W. Moroney, C. H. Reed, N. L. Luban, R. H. Wang, T. E. Eling, R. Childs, T. Ganz, S. F. Leitman, S. Fucharoen and J. L. Miller (2007). "High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin." Nat Med **13**(9): 1096-1101.

Tanno, T., P. Porayette, O. Sripichai, S. J. Noh, C. Byrnes, A. Bhupatiraju, Y. T. Lee, J. B. Goodnough, O. Harandi, T. Ganz, R. F. Paulson and J. L. Miller (2009). "Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells." Blood **114**(1): 181-186.

Teixido, L., B. Carrasco, J. C. Alonso, J. Barbe and S. Campoy (2011). "Fur activates the expression of Salmonella enterica pathogenicity island 1 by directly interacting with the hilD operator in vivo and in vitro." PLoS One **6**(5): e19711.

Torti, F. M. and S. V. Torti (2002). "Regulation of ferritin genes and protein." Blood **99**(10): 3505-3516.

Travier, L. and M. Lecuit (2014). "Listeria monocytogenes ActA: a new function for a 'classic' virulence factor." Curr Opin Microbiol **17**: 53-60.

Uchiyama, A., J. S. Kim, K. Kon, H. Jaeschke, K. Ikejima, S. Watanabe and J. J. Lemasters (2008). "Translocation of iron from lysosomes into mitochondria is a key event during oxidative stress-induced hepatocellular injury." Hepatology **48**(5): 1644-1654.

Ullrich, H. J., W. L. Beatty and D. G. Russell (2000). "Interaction of Mycobacterium avium-containing phagosomes with the antigen presentation pathway." J Immunol **165**(11): 6073-6080.

Vanoaica, L., D. Darshan, L. Richman, K. Schumann and L. C. Kuhn (2010). "Intestinal ferritin H is required for an accurate control of iron absorption." Cell Metab **12**(3): 273-282.

Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland and J. Kreft (2001). "Listeria pathogenesis and molecular virulence determinants." Clin Microbiol Rev **14**(3): 584-640.

Viatte, L. and S. Vaulont (2009). "Hepcidin, the iron watcher." Biochimie **91**(10): 1223-1228.

Vyoral, D. and J. Petrak (2005). "Hepcidin: a direct link between iron metabolism and immunity." Int J Biochem Cell Biol **37**(9): 1768-1773.

Wallace, D. F., C. J. McDonald, L. Ostini and V. N. Subramaniam (2011). "Blunted hepcidin response to inflammation in the absence of Hfe and transferrin receptor 2." Blood **117**(10): 2960-2966.

Wallis, R., D. A. Mitchell, R. Schmid, W. J. Schwaeble and A. H. Keeble (2010). "Paths reunited: Initiation of the classical and lectin pathways of complement activation." Immunobiology **215**(1): 1-11.

Wang, J. and K. Pantopoulos (2011). "Regulation of cellular iron metabolism." Biochem J **434**(3): 365-381.

Weiss, G. (2005). "Modification of iron regulation by the inflammatory response." Best Pract Res Clin Haematol **18**(2): 183-201.

Weiss, G. (2009). "Iron metabolism in the anemia of chronic disease." Biochim Biophys Acta **1790**(7): 682-693.

Wick, M. J. (2011). "Innate immune control of Salmonella enterica serovar Typhimurium: mechanisms contributing to combating systemic Salmonella infection." J Innate Immun **3**(6): 543-549.

Zenewicz, L. A. and H. Shen (2007). "Innate and adaptive immune responses to Listeria monocytogenes: a short overview." Microbes Infect **9**(10): 1208-1215.

Zhang, D. L., T. Senecal, M. C. Ghosh, H. Ollivierre-Wilson, T. Tu and T. A. Rouault (2011). "Hepcidin regulates ferroportin expression and intracellular iron homeostasis of erythroblasts." Blood **118**(10): 2868-2877.

VII. APPENDIX

PBS 10x concentrated

- Sodium chloride - NaCl (Sigma Alrich) …… 90g
- Sodium phosphate dibasic - Na_2HPO_4 (Sigma Alrich) …….. 11,1g
- Potassium phosphate monobasic – KH_2PO_4 (Sigma Alrich) …….. 2g

Above reagents should be dissolved in 1000 ml of distilled water.

1. NON-HEME IRON DETERMINATION IN TISSUES

1.1. Acid mixture

It is composed by 30% of 36,5% hydrochloric acid (Sigma Aldrich Co, St.Louis, MO, USA) and 10% of trichloroacetic acid (Merck, Kenilworth, NJ, USA).

1.2. Working chromogen reagent (WCR)

The solution is prepared by adding 1 volume of the 0,1% chromogen reagent to 5 volumes of saturated sodium acetate (CH_3COONa) and 5 volumes of deonised water.

1.2.1. Saturated sodium acetate

- Sodium acetate – CH_3COONa (Merck, Kenilworth, NJ, USA) …… 300 g
- Deonised water – H_2O (B Braun, Melsungen, Germany) …….. 400 ml

The deonised water is placed to a goblet and 100 g of sodium acetate are added to the goblet. This solution is placed in agitation and the remainder of sodium acetate is then added. The solution is left in agitation with some heat for a while. The final solution should be precipitated.

1.2.2. Chromogen reagent

- Diddonium- 4,7- diphenyl-1,10-phenanthroline dissulfonic acid – $C_{24}H_{14}N_2O_6S_2Na_2$ (Sigma Aldrich Co, St.Louis, MO, USA …… 50 mg
- Thioglycollic acid – $C_2H_4O_2S$ (Merck Kenilworth, NJ, USA) …….. 500 µl
- Distilled water – H_2O (B Braun, Melsungen, Germany) …….. 25 ml

The chromogen reagent is composed by 0,1% of diddonium- 4,7- diphenyl-1,10-phenanthroline dissulfonic acid and 1% of concentrated thioglycollic acid. The $C_{24}H_{14}N_2O_6S_2Na_2$

is added to a 50 ml volumetric flask containing distilled water. Concentrated thioglycollic acid is then added. The final volume is completed by deonised water.

1.3. Working iron standard solution (WISS) [200 µM]

- 36,5% Hydrochloric acid – Hcl (Sigma Aldrich Co, St.Louis, MO, USA) …… 270 µl
- Deonised water – H_2O (B Braun, Melsungen, Germany) …….. 400 ml
- Stock iron standard solution …….. 500 µl

The deonised water is placed in a 50 ml volumetric flask and the 36,5 % hydrochloric acid is added. The stock iron standard solution is also added and the final volume is make up with deonised water.

1.3.1. Stock iron standard solution

- Carbonyl iron powder (Sigma Aldrich Co, St.Louis, MO, USA) ……... 22,3 mg
- 36,5% Hydrochloric acid – Hcl (Sigma Aldrich Co, St.Louis, MO, USA) ……. 1096 µl
- Deonised water – H_2O (B Braun, Melsungen, Germany) …… up to a final volume of 20 ml.

The carbonyl iron powder is added to a 20 ml volumetric flask which contain the 36,5% hydrochloric acid, being dissolved in the acid. This solution should be allowed to stand overnight. The final volume is completed with deonised water.

2. PERLS BLUE STAINING

2.1. Pearls solution composition

- 2% Potassium hexacyanoferrat (II) trihydrate (Merck Millipore, Darmstadt, Germany)…… 0,5g
- 2% Hydrochloric acid (Sigma Aldrich Co, St.Louis, MO, USA) …….. 1350 µL
- Distilled water …….. Up to final volume of 50 ml

2.2. Neutral Red Stain composition

- Neutral Red (Merck Millipore, Darmstadt, Germany)…… 1g
- Distilled water ……100 mL
- Glacial acetic acid (Merck Millipore, Darmstadt, Germany)…… 1mL

3. HEMATOXYLIN EOSIN STAINING

3.1. EOSIN Y SOLUTION 0,5% AQUEOUS

- Eosin Y (Merck Millipore, Darmstadt, Germany …….. 100 mg
- Glacial acetic acid (Merck Millipore, Darmstadt, Germany)…….. 160 µL
- Distilled water ……. 100 ml